Microtubule Assembly and Disassembly at Alkaline pH

C. S. REGULA, J. R. PFEIFFER, and R. D. BERLIN
Physiology Department, University of Connecticut Health Center, Farmington, Connecticut 06032

ABSTRACT Although it is now apparent that the intracellular pH may rise considerably above neutrality under physiological conditions, information on the effect of alkaline pH on microtubule assembly and disassembly is still quite fragmentary. We have studied the assembly/disassembly of bovine brain microtubule protein at alkaline pH in vitro. When microtubules are assembled to a steady state at pH <7 and pH is then made more alkaline, they undergo a rapid disassembly to a new steady state. This disassembly is reversed by acidification. The degree of disassembly is determined largely by the pH-dependence of the critical concentration, which increases five to eight times, from pH 7 to 8. A fraction of assembly-incompetent tubulin is identified that increases with pH, but its incompetency is largely reversed with acidification. Measurements of microtubule lengths are used to indicate that disassembly occurs by uniform shortening of microtubules. A comparison of shortening by alkalinization with dilution suggests that the intrinsic rate of disassembly is accelerated by increasing pH.

The capacity for initiating assembly is progressively lost with incubation at alkaline pH (although some protection is afforded by sulfhydryl-reducing agents). However, direct assembly from depolymerized mixtures is possible at least up to pH 8.3, and the steady state achieved at these alkaline pH values is stable. Such preparations are readily disassembled by cold and podophyllotoxin (PLN). Disassembly induced by PLN is also markedly enhanced at alkaline pH, suggesting a corresponding enhancement of "treadmilling."

The implications of physiological events leading to alkaline shifts of pH for microtubule assembly/disassembly are discussed, particularly in the light of recent hypotheses regarding treadmilling and its role in controlling the distribution of microtubules in vivo.

The microtubules of eukaryotic cells are characteristically in a dynamic state of assembly and disassembly, showing rapid changes in both distribution and number, as a function of cell cycle, cell shape, and diverse surface events. Despite intensive study of microtubule polymerization in vitro, our knowledge of factors that could account for the regulation of microtubule assembly and disassembly in vivo is still fragmentary.

In search of physiological mechanisms of control of microtubule assembly and disassembly, we have considered the role of pH. There is increasing evidence that intracellular pH is rapidly and significantly altered during a variety of physiological processes. For example, the intracellular pH rises by as much as 0.5 unit (from 6.8 to 7.3 or higher) after fertilization of sea urchin eggs (33), and significant alkaline pH shifts have been reported during the acrosomal reaction (31) and during mitosis (10). It is already suggested that these alkaline shifts have important effects on microfilaments. Thus, microfilaments of the sea urchin egg cortex are stabilized at alkaline pH (1), and acrosomal microfilament polymerization may also depend on intracellular alkalinization (34). We proposed that alkaline pH could also affect microtubules, most likely causing their more rapid disassembly. This proposal was based in part on the characteristic absence of microtubules from regions of microfilament aggregation; for example, their absence in the pseudopods of phagocytizing cells, despite a substantial complement of microtubules in other regions of the same cells (3). The simplest hypothesis to explain this reciprocity would be to assume that the same conditions making for microfilament assembly/aggregation promote microtubule disassembly or prevent microtubule assembly. One such condition could be an alkaline pH shift. By this reasoning, the behavior of microtubule protein above pH 7.0 might be critical for understanding microtubule dynamics.

Whereas the pH dependency of assembly has been carefully analyzed between pH 6 and 7, assembly above neutrality has been relatively little studied and disassembly not at all. We shall show that the maximum pH at which assembly can occur is at least pH 8.3 and will indicate why this has not been

THE JOURNAL OF CELL BIOLOGY · VOLUME 89 APRIL 1981 45-53

recognized previously. At alkaline pH, microtubules display quantitatively different properties, including marked enhancement of drug-induced disassembly.

MATERIALS AND METHODS

Microtubule Protein

Microtubule protein was prepared from fresh bovine brain by cyclic assembly/disassembly without glycerol, using slight variations in the procedures of Margolis and Wilson (27). The basic buffer was 20 mM sodium phosphate, 100 mM sodium glutamate, pH 6.75. Assembly medium included 1.0 mM EGTA, 0.5 mM $MgCl_2$, and 1.5 mM GTP, 30°C, 20 min. Disassembly was attained by Dounce homogenization of pelleted microtubules on ice for roughly 40 min. Two or three cycles of assembly/disassembly were employed. Aliquots of the final preparation were stored frozen in buffer at −70°C.

Conditions for Assembly-Disassembly

Aliquots of purified microtubule protein in buffer were prewarmed at 30°C in a constant-temperature chamber. Assembly was initiated by the addition of a × 100 neutralized concentrate of EGTA, Mg, and GTP. pH shifts were induced by addition of Tris base, NaOH, or HCl. These adjustments did not change the ionic strength of sodium concentration by >20%.

SDS polyacrylamide gel electrophoresis, using gradient slab gels of 5–15% with a 3% stacking gel, was performed using the Tris-glycine system of Bryan (6). All samples were boiled for 2 min in SDS immediately before application. The gels were fixed and stained in 0.025% Coomassie Brilliant Blue R, 10% glacial acetic acid, 25% isopropanol, and destained in 10% methanol, 10% glacial acetic acid. For quantitation of gel proteins by scanning, the gels were stained overnight in 0.25% fast green FCF in 50% methanol, 10% acetic acid, and destained in 7.5% acetic acid. Protein was determined by the method of Lowry et al. (22).

Microtubule assembly was followed by turbidity measurements at 350 nm. It has been established that under conditions in which polymer rods are long compared to the wavelength of the incident light, turbidity is a function of the total mass of the scattering particles irrespective of their length (4). This condition was verified over the pH range used. Thus, in all instances abundant microtubules were observed by negative stain and the turbidity or absorbance showed proportionality to logλ (wavelength), and the calculated exponential k was: pH 6.79, −3.36; pH 7.40, −3.14; pH 7.94, −3.14.

The terminology employed is that of Oosawa and Asakura (30), in which "microtubule mass concentration" indicates the total concentration of material in polymeric form and "microtubule number concentration", the concentration of polymeric particles.

Electron Microscopy

For negative staining, a Formvar- and carbon-coated grid was inverted over a drop of sample for 30 s, the excess drained with wet filter paper, and the grid inverted over 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 1 min. The fixed sample was placed over water, then stained with filtered saturated aqueous uranyl acetate, air-dried, and examined in a JEOL 100 CX electron microscope.

To determine microtubule lengths, we photographed and printed the specimens to a final magnification of × 17,800 and measured the individual microtubules with a sonic digitizer (Graf Pen; Science Accessories Corp., Southport, Conn.). The measured lengths of microtubules that extended beyond the edge of the print were multiplied by two, because on the average only half such microtubules remain on the print (16).

For thin-section analyses, the microtubules were pelleted by centrifugation at 100,000 g for 30 min, fixed in 1% glutaraldehyde, and processed as described previously (3).

RESULTS

Microtubule Disassembly by Alkaline Shift of pH

Because several physiological events are known to be accompanied by alkaline pH shifts, we first examined the effect of such shifts in vitro on preassembled microtubules.

As described many times previously, the time-course of microtubule assembly in vitro by light scattering is rapid and slightly sigmoidal, and reaches a plateau that reflects a steady state in which assembly and disassembly are equal. When a microtubule steady state is achieved at the usual "optimal" pH and the pH is then raised, the result is an almost immediate decrease in turbidity followed by a plateau or a very slow decline (Fig. 1, top). In this example (pH shift from 6.8 to 7.6), the initial rate of decay was ~59%/min. This disassembly rate exceeds that induced by colchicine or podophyllotoxin by more than an order of magnitude (25–27) and is comparable to the rate obtained by dilution of assembly mixtures to below the critical concentration for assembly (17). It should be immediately emphasized, however, that whereas dilution may have no physiological counterpart, alkaline pH shifts in vivo are already demonstrated.

Addition of sufficient acid to restore the pH causes a rapid reversal to nearly the original steady-state level (Fig. 1). This reassembled material remains cold labile and microtubules are demonstrable by negative staining in amounts compatible with the optical density (see below).

The chemical species used to induce alkalinization was not critical. Tris base and OH^- (as NaOH) produced equal disassembly. As discussed below, the magnitude of disassembly was a function of pH and total protein concentration.

Assembly at Alkaline pH

When assembly is allowed directly at alkaline pH (bottom curve, Fig. 1), a steady-state plateau is achieved but at a slightly lower level then initially obtained by disassembly from a more acid to the same alkaline pH. (Continued incubation of the disassembled preparation leads finally to the same level [data not shown].) Acidification of the alkaline assembled preparation to pH 6.8 leads to rapid assembly (bottom curve, Fig. 1). After these pH regimens, ~90% of the assembled microtubules are cold labile.

In general, the kinetics of microtubule assembly conform to the predictions of a condensation polymerization mechanism (16, 30). In particular, there is a critical concentration (C_r) of free tubulin dimers below which no polymers are formed, and which is in equilibrium with polymers at all higher concentrations.

We find C_r is strongly pH-dependent. C_r was determined by two methods: extrapolation of steady-state turbidity values obtained at different protein concentrations to zero turbidity, and measurement of the supernatant protein concentration

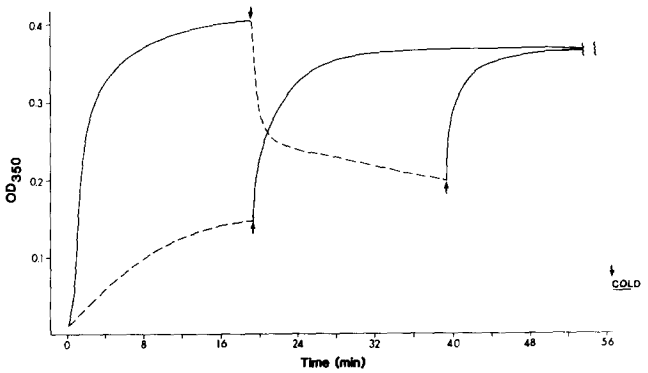

FIGURE 1 Microtubule assembly and disassembly at different pH values. The protein solution was incubated for 2 min at 30°C before initiation of assembly by Mg-GTP. The arrows indicate changes in pH induced by addition of Tris base (arrow down) or HCl (arrow up). Solid line indicates more acid condition, pH 6.65; broken line indicates more alkaline, pH 7.63. Total protein: 3 mg/ml.

after pelleting the microtubules assembled at various total protein concentrations.

Extrapolations of turbidities for a pH series using a single, twice-cycled tubulin preparation are shown in Fig. 2*A*. The intercepts are plotted as a function of pH in Fig. 2*B*. An eight-fold increase in C_r was seen, from pH 6.35 to 7.75. A significant increase in C_r with pH was reported by Doenges et al. (7), using a similar extrapolation. Increasing pH thus should force microtubule disassembly until the concentration of free tubulin reaches the new C_r and a new steady state is achieved.

The slopes of the linear regression equations fitted by least squares to the data at each pH are given in the legend to Fig. 2*A* and are slightly different, decreasing progressively with increasing pH. Thus, the differences between C_r and the total protein concentrations also increase at a given OD. However, because the microtubule mass concentration is proportional to the OD, these differences imply that a decreasing fraction of total protein is competent to assembly at increasing pH. Therefore, the total protein concentrations cannot be used directly to compare C_r at different pH values. To correct for this effect, we normalized the apparent critical concentrations to the condition at pH 6.35 by multiplying C_r by the ratio of the slope at any pH to the slope at 6.35. This essentially eliminates "incompetent" protein in excess of that present at pH 6.35. The values obtained are plotted in Fig. 2*B*, using open circles. A similarly strong dependence of C_r on pH is seen.

To obtain the C_r in terms of the absolute concentration of competent microtubule protein, we assembled microtubules at varying total protein concentration to the steady state, pelleted the microtubules, and measured the supernatant protein concentration. These were then extrapolated back to zero total protein. The results for assembly at pH 6.53 and 7.50 are shown in Fig. 2*C*. The extrapolated values for C_r are 0.18 mg/ml at pH 6.53 and 0.79 mg/ml at pH 7.50. This five-fold increase is consistent with the results obtained from turbidity measurements. In addition, because the C_r should remain constant irrespective of the total assembled mass, any supernatant protein in excess of C_r is essentially incompetent of assembly. The slope of the supernatant extrapolation (see legend) at pH 7.5 is nearly double that at pH 6.5, indicating double the fraction of incompetent protein. In this preparation, cycled three times and stored frozen at −70°C for 1 wk before use, this fraction was 13%. Under our conditions of storage, the decay of competency was roughly 5%/wk.

We emphasize that the greater degree of assembly incompetence at alkaline pH is largely reversible. As shown in Fig. 1, a shift to more acid pH leads to assembly that is nearly equal to the assembly obtained in solutions that had not been exposed to alkaline pH.

It is apparent that the absolute change in steady-state level for any pH change is dependent on the total microtubule protein concentration. In essence, pH shifts correspond to vertical movements between the family of lines represented in Fig. 2*A*. Thus, the lower the protein concentration, the more exaggerated the relative change in the steady-state level of polymerization.

Depolymerization

To analyze the pathway of depolymerization after a shift to more alkaline pH, we determined the microtubule-length distribution. Fig. 3*A* is a histogram of lengths during steady-state assembly/disassembly at pH 6.8. In this example a shift to pH 7.6 by addition of Tris base led to a new steady state with an assembled mass (proportional to OD) some 41% less. Fig. 3*B* is a histogram of microtubule lengths at the new pH value. The mean microtubule length was reduced by 19%. This small degree of shortening suggests at once that fragmentation does not occur as a result of alkalinization. Further analysis showed that the length distributions were entirely consistent with microtubule shortening by a zero-order process (i.e., proportional to the number of microtubule ends and not to the length of the microtubule). Specifically, we tested the hypothesis that the decrease in microtubule mass was caused by a shortening of all microtubules at the same rate. The histogram (Fig. 3*C*) was constructed from the data represented in Fig. 3*A* by shortening all measured microtubules an equal amount. The total length decrease for all microtubules was calculated from the pH-associated decrease in polymer mass concentration. Where the shortened "length" exceeded the actual microtubule length, the difference was distributed over the remaining microtubules, which were additionally shortened. Iteration of this procedure quickly converged to a distribution for all remaining microtubules.

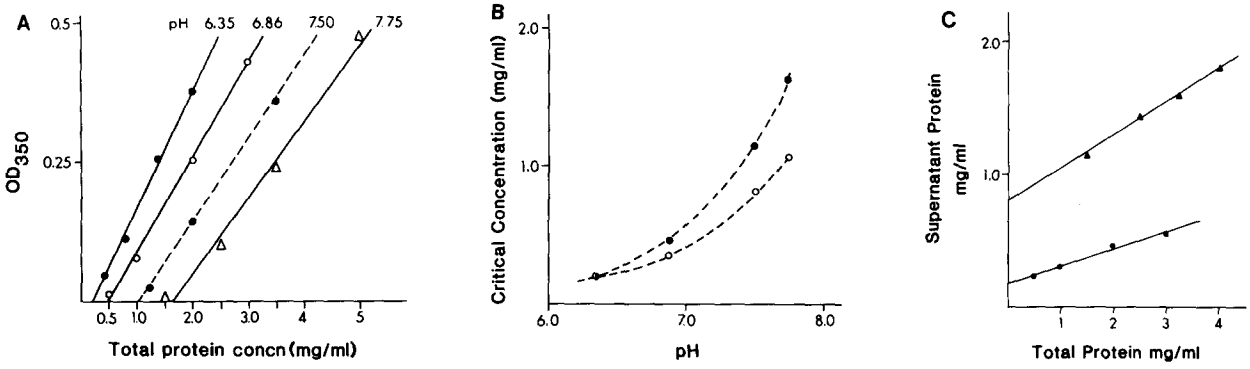

FIGURE 2 Critical concentration (C_r) for microtubule assembly as a function of pH. (*A*) Plots of the turbidity at its plateau (steady state) for microtubules assembled at different pH as a function of protein concentration. The phosphate-glutamate buffer was adjusted with HCl or Tris and the pH measured at the conclusion of the experiments. The ionic strength was between 0.08 and 0.1. The lines were drawn according to least-square fit of the data, $r^2 = .99$. The ratios of the slopes for pH 6.35:6.85:7.50:7.75 were 1: 0.81:0.72:0.65. (*B*) A plot of C_r as determined from the *x*-intercepts of *A* as a function of pH. The upper curve is taken directly from the figure. The lower line is calculated accounting for the difference in slopes as explained in the text. (*C*) Supernatant protein remaining after centrifugation of microtubules assembled at varying total protein concentration. The *y*-intercepts correspond to the C_r in the absence of nonpolymerizable protein. ▲, pH 7.50; ●, pH 6.53.

The agreement with the measured distribution, Fig. 3 *B*, is remarkable: there is no statistically significant difference by *t* test and the calculated mean microtubule lengths are nearly identical to the observed values. The fall-off in rate of disassembly probably occurs because of a diminishing number of disassembly sites resulting from the complete disassembly of the shorter microtubules as described by Johnson and Borisy (16) for the kinetics of cold disassembly.

In this experiment, a new steady state was achieved very quickly and maintained for the next 45 min (the longest period followed). The rather short mean length of microtubules indicated a high number concentration that would favor any zero-order process. In some experiments microtubules assembled to approximately the same OD were found to undergo a rapid initial decrease on alkalinization, followed by a slow decline (see Fig. 1 and *inset*, Fig. 4). In this case, length histograms revealed a greater mean length (Fig. 4*A*) and a more gradual decrease in length (Fig. 4*B* and *C*), which is also consistent with a hypothetical simple shortening mechanism (theoretical histograms not shown).

Acceleration of Disassembly at Alkaline pH

Thus far, it would appear that the fall in steady-state level after a shift from acid to alkaline pH can be explained by a shortening of microtubules until disassembly is sufficient to raise the free microtubule protein to the critical concentration. However, this implies nothing about the rate of disassembly per se. The rapidity of change on pH shift suggested that the disassembly rate was also greatly accelerated.

To rest this possibility more directly, we compared microtubule shortening from pH shift with shortening from rapid dilution of microtubules assembled to a steady state. The two cases are highly analogous in that in both conditions the free microtubule protein is decreased below the critical concentration: in the alkaline shift, C_r is raised; in dilution at the same pH, C_r remains constant, while the free microtubule protein concentration is lowered. Samples were taken for determination of microtubule length before and 60 s after pH shift/dilution. Understandably, the time of the fixation could not be precisely known. However, all samples were treated identically so that microtubule length distributions could be directly compared. After dilution at the same pH, no shortening (in fact, a small lengthening) was observed. Although rapid shortening might be expected based on the OD changes as reported by Karr and Purich (17), our result is consistent with the statistical variation in length measurement reported by Bergen and Borisy (2), whose data indicate that changes in microtubule length cannot be detected reliably over a 60-s interval. By contrast, after the same interval a pH shift leads to a marked 30% shortening that is increased to 48% by dilution. These much larger changes in length induced by alkalinization indicate an increase in the intrinsic rate of disassembly. We emphasize that the acceleration of disassembly is not the result of an increased number of microtubule ends. As discussed above, the distribution of microtubule lengths after alkalinization is consistent with a simple shortening mechanism. Moreover, based on the fall in OD or polymer mass concentration, and the relatively smaller decrease in the mean microtubule length, it is evident that the concentration of ends is actually decreasing during alkaline disassembly.

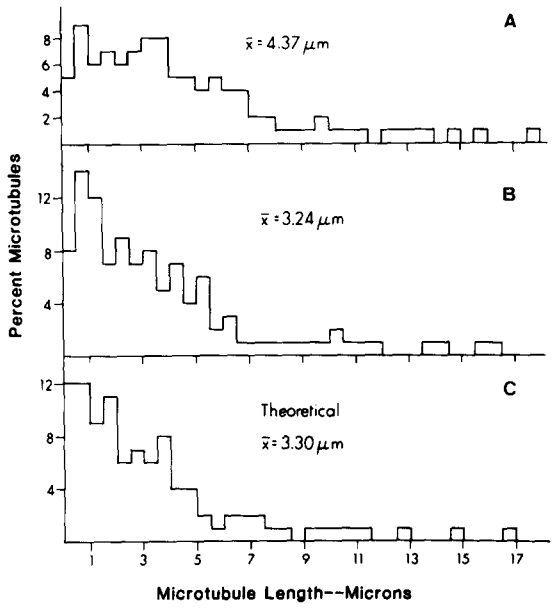

FIGURE 3 Histograms of microtubule lengths before and after disassembly associated with alkaline pH shift. The $\bar{x}$ corresponds to mean microtubule length. Fig. 3 illustrates an experiment in which the shift led to an immediate plateau in optical density. (*A*) Histogram sampled from steady-state assembly at pH 6.8 1 min before alkalinization. (*B*) Histogram 1 min after shift to 7.6. (*C*) A theoretical distribution of lengths based on the initial distribution *A* and equal shortening of all microtubules as described in the text.

Nucleation and Kinetics of Assembly

A comparison of the length distribution of microtubules directly assembled at alkaline pH with that of microtubules at the same pH derived from a population assembled initially at a more acid pH reveals that, despite the slightly lower polymer mass concentration, the mean microtubule length is significantly greater in the alkaline-assembled preparation. Histograms of the microtubule length distributions obtained at alkaline pH are shown in Fig. 5. These histograms are directly comparable to those of Fig. 4, obtained in parallel with the same protein preparation assembled at pH 6.8. After alkalinization of the pH 6.8–assembled protein (Fig. 4), the OD falls to ~0.105. On direct assembly at the same alkaline pH, the

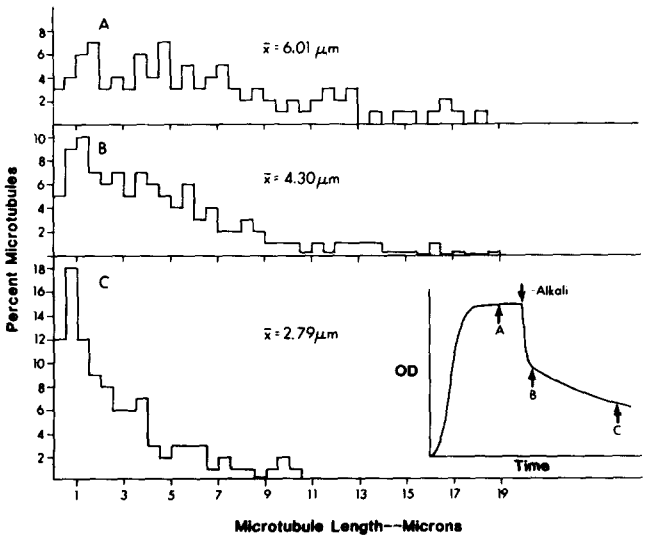

FIGURE 4 Progressive shortening of microtubule lengths in an experiment in which the new steady state at alkaline pH was established more gradually. *A* is the steady-state distribution. *B* was obtained 1 min after shift to pH 7.8; and *C*, 9 min after shift.

OD plateaued at ~0.085 (Fig. 5). Because the mean length is greater for microtubules assembled at alkaline as compared with acid pH at roughly the same assembled mass, it follows that the microtubule number concentration is lower with alkaline assembly. Thus, the nucleation of microtubule assembly at alkaline pH is also impaired. The effect of pH on the stability of "initiation factors" will be discussed below.

We did not study the early kinetics of assembly. However, comparison of Fig. 5 *A* and *B* (measurements taken at roughly 50% and 100% [OD = 0.046 and 0.085], respectively, of the steady-state level of assembly) shows that the increase in OD can be accounted for entirely by lengthening existing microtubules. Thus, nucleation must have been complete by ~5 min.

The rates of assembly as a function of pH were treated only briefly. As expected, the rate increased with increased total protein concentration. This was shown roughly by plots of the log of the rate of assembly at its maximum as a function of total protein concentration. The data for assembly at pH 6.35 and 7.75 are presented in Fig. 6. Rapid rates at alkaline pH values are obtainable but at higher total protein concentrations.

The rates of elongation may be approximated from the length histograms and OD measurements. We assume that all nucleation is complete before attainment of the maximum assembly rate (steepest slope of assembly curve) and, thus, that the microtubule number concentration is the same as at steady state (proportional to the steady-state OD, i.e., mass/microtubule length) and that the mean microtubule length at that point can be calculated as a fraction of the mean steady-state length: (OD at maximal rate/OD at steady state) × steady-state length. Relative rates of elongation may then be determined from this number concentration and the steepest slope. From the data of Figs. 4 and 5, for example, it may be calculated that the number concentration at pH 6.8 is five times that at 7.89. At the point of most rapid net assembly, the slope at pH 6.8 is roughly 7.5 times as great. Consequently, the difference in assembly rate is largely a result of the number of sites available for growth. Although this calculation is very rough, it suggests that the intrinsic assembly rate is less sensitive to pH than is disassembly. This may describe the summed behavior of both ends of the microtubule and obviously does not account for potential differences resulting from polarized growth.

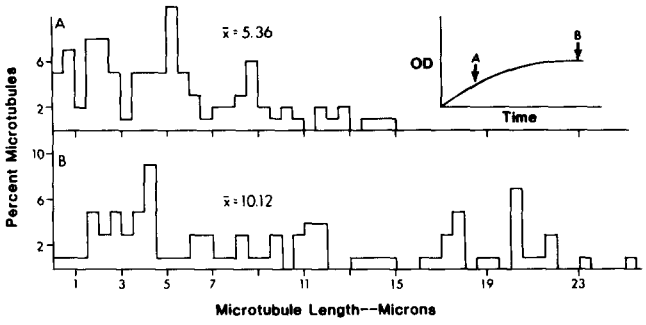

FIGURE 5 Histograms of microtubule lengths during assembly at pH 7.89. Sample *A* was taken at an OD of 0.046 and *B* at 0.085. The implication of the correspondence between relative length and OD is that by 5 min nucleation is complete, microtubule number concentration is constant, and the increase in microtubule mass is the result of microtubule elongation. This protein preparation was also used to obtain the data represented in Fig. 4. Even at a somewhat lower OD than the disassembled preparation shown in Fig. 4 at *C*, the mean microtubule length is considerably greater in the microtubule preparation assembled directly at an alkaline pH.

Microtubule Disassembly by Podophyllotoxin (PLN) Is pH-dependent

We first established that PLN completely blocks assembly at all pH values (6.2–8.3). The effect of PLN on preassembled polymers was then tested. Microtubules were assembled at various pH values to the steady state. PLN was then added and the decay rate determined from the initial rate of decrease in turbidity. Fig. 7 shows the turbidity curves for a typical experiment. Fig. 8 gives the fractional decrease expressed as a percentage of the steady-state level plotted against pH. The PLN-induced disassembly approaches zero near pH 6.0 (extrapolation actually yields pH 5.80) and is roughly 6%/min at pH 8.0. The experiment analyzed gives a conservative estimate of the effect of PLN disassembly. Rates of 15–20%/min. at pH 8.0 were commonly observed.

The differential action of PLN as a function of pH is not related to the absolute steady-state level of polymerization, which at the same total protein concentration was shown above to be inversely related to pH. When assembly is allowed at various protein concentrations and PLN is then added, the

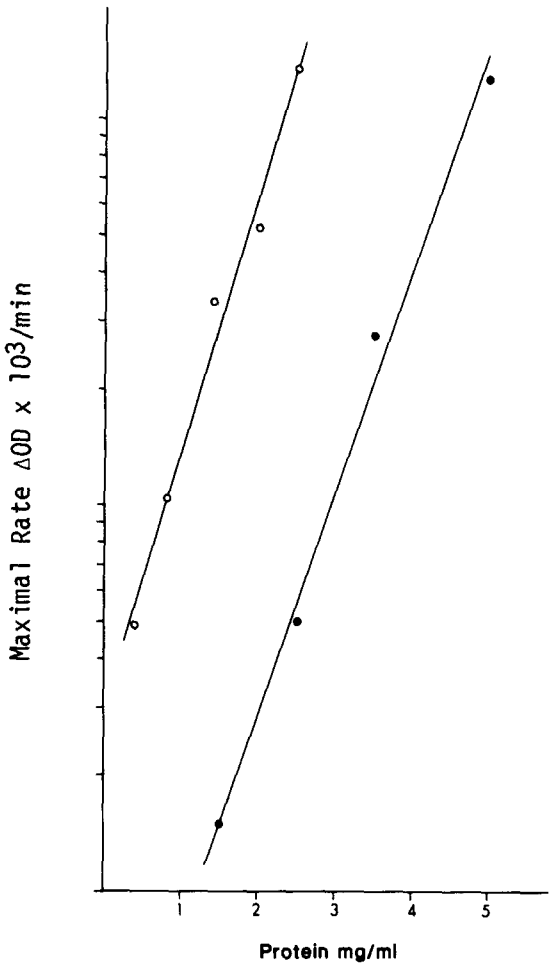

FIGURE 6 Rate of microtubule assembly as a function of protein concentration at pH 6.35 (○) and pH 7.75 (●). The rates were obtained at the steepest slopes of the rising OD assembly curves. The data were fitted by least squares to the equation, log y = log a + bx. For pH 6.35, log(OD × 10^3/min) = −1.11 + 1.52 C, r^2 = 0.96; for pH 7.75, log(OD × 10^3/min) = 1.51 + 1.20 C, r^2 = 0.99 where C = protein concentration in milligram per milliliter. The slopes differ by only 15% but are displaced with respect to protein concentration.

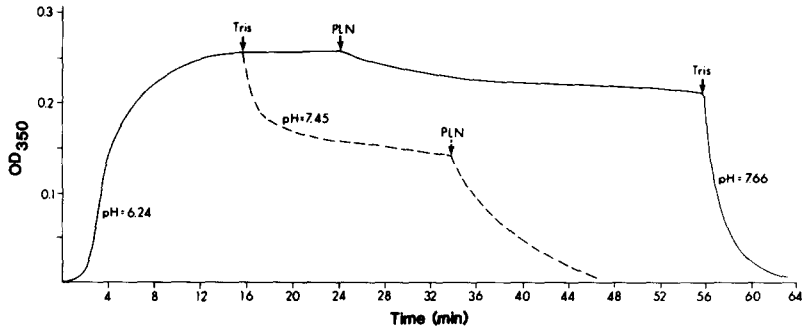

FIGURE 7 Effect of podophyllotoxin (PLN) at different pH values. In this example, microtubule protein was 2.5 mg/ml assembled at pH 6.24 to a steady state. 50 μM PLN was added directly at pH 6.24 or after alkalinization to pH 7.45. The much steeper slope of disassembly at alkaline pH is readily observed, and complete disassembly occurs in 15 min. The order of addition causing rapid and complete disassembly makes no difference as shown by the alkalinization of the PLN-treated sample (right).

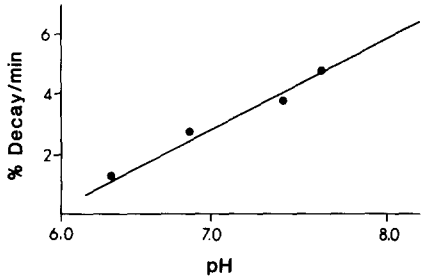

FIGURE 8 Summary graph of PLN disassembly as a function of pH. In these experiments, assembly was at pH 6.24. Aliquots were then alkalinized to varying pH by the addition of small volumes of concentrated Tris. After 6 min when a new steady state was nearly established, 50 μM PLN was added as a × 100 concentrated solution in ethanol and the initial rate of disassembly measured. The results given as percent decrease of the initial OD are plotted as a function of the final measured pH. The line is drawn by least square fit: % decay/min = 13.97 + 2.41 (pH), r^2 = 1.0. It should be emphasized that PLN blocks assembly at all pH. The experiment illustrated was performed with one tubulin preparation. Some variation between tubulin preparations was encountered, but the relative percent decay as a function of pH was approximately the same, though usually greater in magnitude than illustrated.

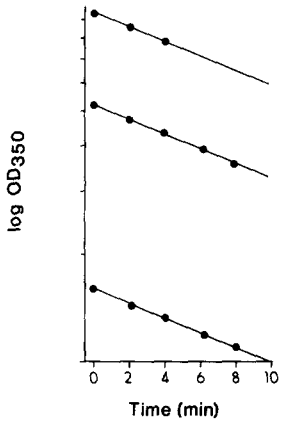

FIGURE 9 Disassembly rates at constant pH (7.75) at three total protein concentrations: (top) 5 mg/ml, (middle) 3.5 mg/ml, and (bottom) 2.5 mg/ml. Microtubules were assembled directly at pH 7.75 (no pH shift), and PLN was added after attainment of the steady state. The decay rates are nearly independent of total protein concentration and are first order, with a rate constant of ~.057/min. As explained in the text, these data are not directly comparable to those of Fig. 8, which were obtained after alkalinization of a different pH 6.24-assembled preparation. As discussed, the number concentration of the latter is significantly higher than that of the preparation assembled directly at alkaline pH.

calculated rate of decay is essentially identical at a given alkaline pH for all protein concentrations (Fig. 9). This is to be anticipated, of course, if PLN acts on microtubule ends and if the number of ends is proportional to total protein concentration. Because nucleation is impaired at alkaline pH and thus the microtubule number concentration is reduced at alkaline pH, it is clear that the increasing rates of depolymerization after addition of PLN cannot be the result of increasing the number of ends available for disassembly.

PLN-induced disassembly is nevertheless dependent on the microtubule number concentration as expected. No detailed study of this was made, but it is evident from the following (see Fig. 1). When, instead of an acid (at the arrows pointing upward), PLN was added, rapid disassembly occurred from both the direct-alkaline-assembled and alkaline-shifted preparations. However, in one experiment at pH 7.8, for example, the decay rate of the former preparation was 11%/min and of the latter, 18%/min. From the analysis of Figs. 4 and 5, it is clear, however, that the number concentration of the direct-alkaline-assembled microtubules is significantly less and, thus, the lower decay rate is explicable in terms of the number of "ends" available.

Composition of Microtubules Assembled at Various pH Values

The increase in C_r, impaired initiation, and susceptibility to disassembly by colchicine (14, 32) are formally similar to microtubules assembled from purified 6S tubulin; i.e., without microtubule-associated proteins (MAPs). It seemed possible that these associated proteins might not be coassembled at alkaline pH. To test this possibility, we examined the protein composition of microtubules assembled at normal and alkaline pH by SDS PAGE and by electron microscopy. The analysis was performed on microtubules pelleted by centrifugation. When assembly was blocked by PLN under normal conditions of incubation, little protein was pelleted (<5% of total), irrespective of pH. Thus, the protein analyzed was indeed associated with microtubules. Fig. 10 is a photograph showing the fast-green-stained electropherogram patterns for microtubules assembled at various pHs between 6.3 and 7.8. No differences are apparent. Densitometric scans (not shown) confirmed this impression. MAPs constituted roughly 28% of the total protein

assembled, which is in the reported range for bovine brain microtubule protein prepared in the absence of glycerol (27). The association of MAPs with microtubules at all pH values was confirmed by electron microscopy of thin-sectioned pellets (Fig. 11). The characteristic filamentous coat and separation of microtubules at pH 6.5 reported by others (31) was also found at pH 7.8.

Stability of Microtubule Protein at Alkaline pH

Gaskin et al. (9) indicated that cold-reversible assembly was possible in only a narrow pH range—and was absent above neutrality. However, a total protein concentration of only 2 mg/ml was used in their experiments, which we show here begins to approach the critical concentration at higher pH. In addition, in their experiments cold reversal was attempted after incubation for 1 h at 25° or 37°C. Indeed, we find that tubulin gradually loses its capacity for assembly on incubation at alkaline pH. Fig. 12 depicts the progressive decrease in assembly competence when microtubule protein was incubated at pH 7.5 at 30°C. Because, in general, disulfide formation occurs more readily at alkaline pH and tubulin is affected by sulfhydryl oxidants (19), it seemed possible that reducing agents might prevent the loss of assembly competence. In fact, at modest elevations of pH, dithiothreitol did retard inactivation (Fig. 12, top line). At higher pH, however, inactivation was more rapid and was unaffected by sulfhydryl-reducing agents. For example, at pH 8.0, assembly competence was lost at the rate of roughly 1–3%/min. However, the pH-sensitive step appears to be initiation of assembly. Thus, microtubules assembled to a steady state at pH values as high as 8.3 show no disassembly with continued incubations of 40–50 min (the longest followed). Such assembled microtubules remain sensitive to disassembly by PLN. The determination of C_r (above) as a function of pH was done over a time period during which only a small degree of inactivation occurred (10%). This inactivation is not to be confused with the reversible incompetence revealed in the differing slopes of Fig. 2*A*.

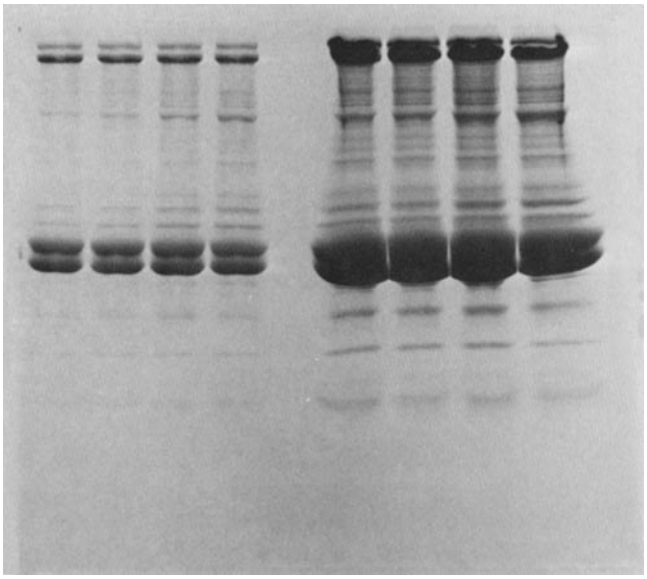

FIGURE 10 Fast-green-stained SDS PAGE slab gel proteins solubilized from microtubules that had been assembled at varying pH from × 3 cycled microtubule protein. Two electropherograms of gels loaded with samples of 50 or 250 μg of protein respectively. From left to right: pH 6.29, 6.79, 7.27, and 7.81.

DISCUSSION

The properties of microtubule polymerization in the alkaline pH range have been relatively neglected. Indeed, assembly has been reported absent above pH 7.0 or 7.2 (9, 12, 21, 29). However, assembly at more alkaline pH has been found (7) and shown to be enhanced by high concentrations of organic sulfates (15). It is clear from our studies that the limited assembly observed can be explained in part by the increase in the critical concentration with increasing pH and by loss of capacity for initiation with prolonged incubation at alkaline pH. Our results indicate that significant polymerization of microtubule protein occurs up to pH 8.3 (and perhaps higher). The slope of the ln $1/c$ vs. $-\ln a_{H+}$, where c is the protein concentration, of 0.62 ± 0.29 is in fair agreement with the

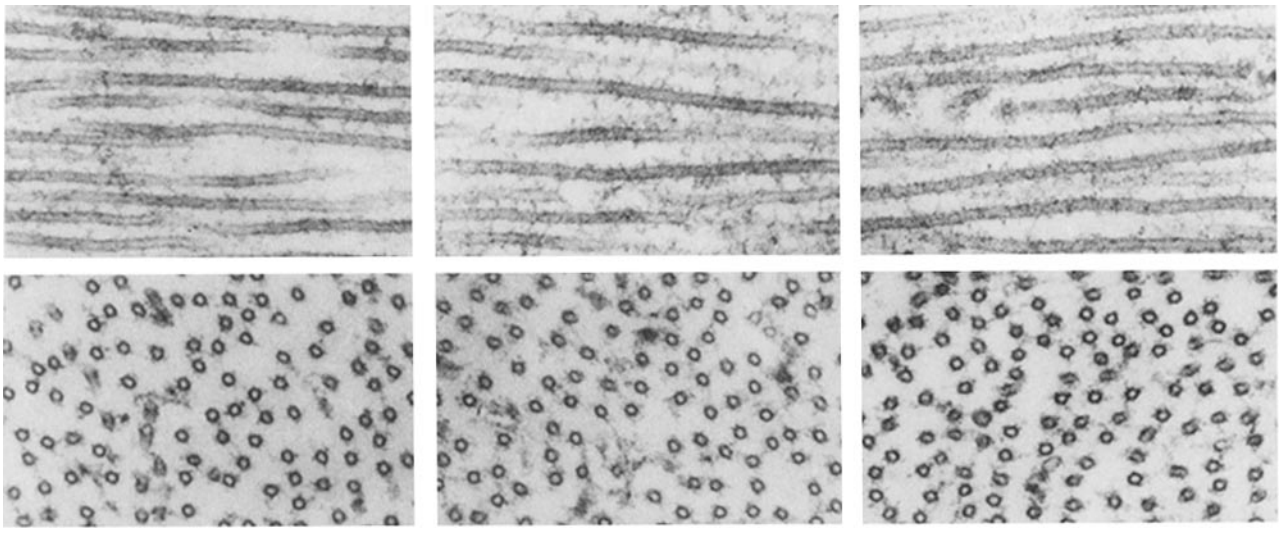

FIGURE 11 Electron microscopy of thin-sectioned microtubule pellets assembled at pH 6.8 and 7.8. Top panels show areas cut parallel to longitudinal microtubule axes and bottom panels are cross sections. Left: pH 6.8 assembly; center: pH 6.8 assembly followed by pH shift to 7.8 for partial disassembly; right: pH 7.8 assembly. A fuzzy coat is observed under all conditions and the microtubule cross sections are well separated.

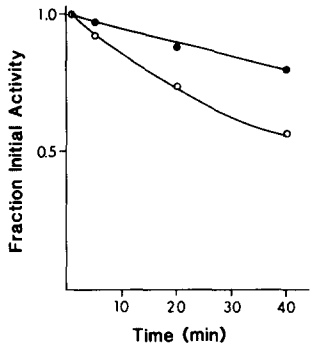

FIGURE 12 Loss of assembly competence at pH 7.50 with (●) or without (○) 5 mM dithiothreitol (DTT) as a function of time. In these experiments microtubule protein at pH 6.8 with or without DTT was prewarmed to 30°C for 5 min. This DTT made no difference to assembly at this pH over the next 40 min. At 0 time the solution was brought to pH 7.5 by addition of concentrated Tris base. At various subsequent intervals, assembly was initiated by the addition of Mg-GTP. The results are expressed as a fraction of the steady-state plateau achieved with simultaneous addition of Tris and Mg-GTP.

value (0.86 ± .15) obtained by Lee and Timasheff (20) for bovine brain tubulin assembled in the presence of glycerol. Assembly at appropriately high protein concentration above the C_r follows a rapid time-course that is virtually identical to that obtained at the usual pH of assembly. When the data of Fig. 2*A* are plotted according to the linked function relations of Wyman (37), no discontinuity is observed above neutrality.

We focused particularly on the effects of shifts in pH on microtubule dynamics. We show that increasing pH leads to rapid disassembly by shortening of microtubules and that the intrinsic rate of disassembly is greatly accelerated. The intrinsic assembly rate is probably decreased as well. These results imply that the state of microtubule assembly is extremely sensitive to changes in intracellular pH, particularly above neutrality.

These properties are likely to be relevant physiologically. Rapid increases in pH have now been documented in several systems, for example, in fertilization (33) and in the acrosome reaction (31, 34). It is likely that a whole range of hormonal and other agents that may utilize cyclic AMP as a second messenger will cause increases in intracellular pH. Thus, Boron et al. (5), in what may be a glimpse into a general mechanism, showed that cyclic AMP markedly enhanced acid extrusion from a perfused muscle cell preparation. What are the likely effects of an alkaline pH shift? To a first approximation, a rise in pH should cause disassembly to a degree dependent on the pH increment and total microtubule protein concentration, as explained above in reference to Fig. 2*A*. Cells with the lowest concentrations might be most affected. The actual situation may be more complicated and may be profitably discussed in terms of Kirschner's (18) recent speculations on the significance of treadmilling (the movement of microtubule subunits from assembly at one end of the microtubule to disassembly at the other).

A necessary consequence of treadmilling as originally formulated by Wegner (35) is that C_r is different and lower at the assembly end as opposed to the disassembly end of the polymer. In Kirschner's view, an important consequence of this difference is that microtubules anchored to centrioles (and hence, perhaps, kinetically inert) at the disassembly end, are in equilibrium with a low free-tubulin concentration, whereas unanchored microtubules are disassembled at the same free-tubulin concentration. In this way, microtubule networks might be centrally directed. We show here that the sensitivity of microtubules to disassembly by PLN is markedly pH-dependent. According to Margolis and Wilson (26), this rate of disassembly from the steady state at high concentrations of PLN is a measure of treadmilling. Treadmilling is thus positively correlated with pH. Consequently, with increasing pH not only is C_r raised but the difference in C_r at the assembly end as compared with that at the disassembly end will be increased. This would have the effect, if Kirschner's view is correct, of more effectively suppressing free microtubules. Harris et al. (13) show that even in the large *Strongylocentrotus purpuratus* egg, fertilization leads initially to microtubule growth almost exclusively from centrioles. This period is clearly one of pronounced cytoplasmic alkalinization (33). At subsequent stages, the microtubules of the fertilized *S. purpuratus* egg undergo a wave of disassembly that seems to originate centrally. This disassembly may be correlated with a wave of increased free calcium (11) that will promote disassembly (24, 36). However, localized pH changes are also possible, driven by proton sinks or other metabolic events. Local changes would be promoted in regions of a high surface (ion pumping) area to volume ratio. The pseudopod of phagocytic cells is an example of a geometry of this kind.

We have previously shown that pseudopods, though rich in microfilaments, are devoid of microtubules: the latter underlie the microfilamentous extension (3). Localized proton efflux in the pseudopod cup could lead to intracellular alkalinization and the inhibition of microtubule assembly or rapid microtubule disassembly in the underlying cytoplasm. Nuccitelli et al. (28) demonstrated a current flow (flow of positive charge) into the tail and out of the pseudopods of amoebae. Though calcium seemed to carry most of the inward current, the ionic species flowing outward were not identified. The currents measured were sufficiently large that if even a small part were carried by protons, a significant rise in local intracellular pH might occur. This argument, although highly speculative, makes plausible the idea that a local pH change might be generated by the pseudopod membrane to a degree sufficient to explain the absence of microtubules within it.

The pH dependency of microtubule disassembly also may explain some of the variability among different cell types and systems in response to PLN or colchicine. With intracellular pH set low, microtubule assembly could become highly resistant to PLN, and vice versa.

In many respects, microtubules behave above neutral pH as though deficient or devoid of associated protein (MAPs): C_r is elevated, rates of assembly and initiation are impaired, and susceptibility to disassembly by PLN is increased. However, direct analysis shows that there are no major differences in the proteins associated with microtubules assembled over the pH range of 6.3–7.8. In fact the high proportion of MAPs in the assembled microtubules at all pHs suggests that binding sites are saturated. Thus it is apparent that the simple colocalization of tubulin and MAPs by specific immunofluorescence does not define the kinetic behavior of the associated microtubules.

Several aspects of polymerization at alkaline pH require further study. In particular, the apparently defective nucleation of assembly at alkaline pH could correspond to the predominance of an 18S over 30S tubulin multimer at higher pH (7.4–8.2) described by Marcum and Borisy (23) and by Doenges et al. (7). Galella and Smith (8) recently indicated that above pH 8.2 MAPs were irreversibly denatured. We did not explore this

higher pH range but we observed an unexplained variation in the stability of microtubule protein near pH 8.0, perhaps because of a subtle defect in MAP-tubulin interaction or depletion of a quantitatively minor MAP that is not obvious from the gel pattern. We show that the major irreversible effect of alkaline pH is a defect in nucleation. Once microtubules are assembled, there is little denaturation. The molecular pH-sensitive target has not been identified.

The increased rate of PLN-induced disassembly with pH also demands closer investigation. The PLN-disassembly rates at alkaline pH greatly exceed those previously reported, including the recently reported sensitization by ATP (27). If, indeed, PLN disassembly is a measure of treadmilling, its role in any process, including its significance for the stability of anchored microtubules already discussed, will be enhanced at alkaline pH. The recent experiments of Bergen and Borisy (2) employing direct length measurement of microtubules assembled onto axonemes demonstrated appreciable assembly and disassembly at both "ends" of the microtubules, and only limited theoretical treadmilling. However, Bergen and Borisy were careful to indicate that their measurements were made under only one set of experimental conditions, which included a pH of 6.75. Thus, it will be interesting to determine whether the mechanisms of PLN action and of the polarity of microtubule assembly and disassembly are the same at more alkaline pH.

It is clear that the role of pH in microtubule dynamics in living cells bears careful investigation. Previous data on intracellular pH have not been gathered with the analysis of microtubule assembly in mind. Thus, our discussion of physiological relevance is clearly inconclusive. A significant need exists for simultaneous evaluation of intracellular pH and microtubule assembly with particular regard for the formidable problem of evaluating pH within local regions. Because of its significant effects on every aspect of microtubule polymerization, particularly disassembly, C_r, and perhaps treadmilling, intracellular pH will likely prove a critical parameter in cytoskeletal regulation.

This work was supported by National Institutes of Health grants CA15544 and HL23192.

Received for publication 18 August 1980, and in revised form 3 December 1980.

REFERENCES

1. Begg, D. A., and L. I. Rebhun. 1979. pH regulates the polymerization of actin in the sea urchin egg cortex. *J. Cell Biol.* 83:241–248.
2. Bergen, L. G., and G. G. Borisy. 1980. Head-to-tail polymerization of microtubules in vitro. *J. Cell Biol.* 84:141–150.
3. Berlin, R. D., and J. M. Oliver. 1978. Analogous ultrastructure and surface properties during capping and phagocytosis in leukocytes. *J. Cell Biol.* 77:789–804.
4. Berne, B. J. 1974. Interpretation of the light scattering from long rods. *J. Mol. Biol.* 89: 755–758.
5. Boron, W. F., M. M. Russell, and M. S. Brodwick. 1978. Influence of cyclic AMP on intracellular pH regulation and chloride fluxes in barnacle muscle fibers. *Nature (Lond.).* 276:511–513.
6. Bryan, J. 1974. Biochemical properties of microtubules. *Fed. Proc.* 33:152–157.
7. Doenges, K. H., S. Bierdert, and N. Paweletz. 1976. Characterization of a 20S component in tubulin from mammalian brain. *Biochemistry.* 15:2995–2999.
8. Galella, G., and D. B. Smith. 1979. Stability of microtubule protein over the pH range: 6.9–9.5. *Can. J. Biochem.* 57:1368–1375.
9. Gaskin, F., C. R. Cantor, and M. L. Shelanski. 1974. Turbidimetric studies of the *in vitro* assembly and disassembly of porcine neurotubules. *J. Mol. Biol.* 89:737–758.
10. Gerson, D. F., and A. C. Burton. 1977. The relation of cycling of intracellular pH to mitosis in the acellular slime mould *Physarum polycephalum. J. Cell. Physiol.* 91:297–304.
11. Gilkey, J. C., L. F. Jaffe, E. Ridgway, and G. T. Reynolds. 1978. A free calcium wave traverses the activating egg of the medaka, *Oryzias latipes. J. Cell Biol.* 76:448–466.
12. Haga, T., T. Abe, and M. Kurokawa. 1974. Polymerization and depolymerization of microtubules *in vitro* as studied by flow birefringence. *FEBS (Fed. Eur. Biochem. Soc.). Lett.* 39:291–295.
13. Harris, P., M. Osborn, and K. Weber. 1980. Distribution of tubulin-containing structures in the egg of the sea urchin *Strongylocentrotus purpuratus* from fertilization through first cleavage. *J. Cell Biol.* 84:668–679.
14. Herzog, W., and K. Weber. 1977. *In vitro* assembly of pure tubulin into microtubules in the absence of microtubule-associated proteins and glycerol. *Proc. Natl. Acad. Sci. U. S. A.* 74:1860–1864.
15. Himes, R. H., C. S. Newhouse, K. M. Haskins, and P. R. Burton. 1979. Effects of sulfonate anions on the self-assembly of brain tubulin. *Biochem. Biophys. Res. Commun.* 87:1031–1038.
16. Johnson, K. A., and G. G. Borisy. 1977. Kinetic analysis of microtubule self-assembly *in vitro. J. Mol. Biol.* 117:1–31.
17. Karr, T. L., and D. L. Purich. 1979. A microtubule assembly/disassembly model based on drug effects and depolyermization kinetics after rapid dilution. *J. Biol. Chem.* 254:10885–10888.
18. Kirschner, M. W. 1980. Implications of treadmilling for the stability and polarity of actin and tubulin polymers in vitro. *J. Cell Biol.* 86:330–334.
19. Kuriyama, R., and H. Sakai. 1974. Role of tubulin-SH groups in polymerization of microtubules. *J. Biochem. (Tokyo).* 76:651–657.
20. Lee, J. C., and S. N. Timasheff. 1977. *In vitro* reconstitution of calf brain microtubules: effects of solution variables. *Biochemistry.* 16:1754–1764.
21. Lee, Y. C., F. Samson, L. L. Houston, and R. Himes. 1974. The *in vitro* polymerization of tubulin from beef brain. *J. Neurobiol. 5*:317–330.
22. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.
23. Marcum, J. M., and G. G. Borisy. 1978. Characterization of microtubule protein oligomers by analytical ultracentrifugation. *J. Biol. Chem.* 253:2825–2833.
24. Marcum, J. M., J. R. Dedman, B. R. Brinkley, and A. R. Means. 1978. Control of microtubule assembly-disassembly by calcium-dependent regulator protein. *Proc. Natl. Acad. Sci. U. S. A.* 75:3771–3775.
25. Margolis, R. L., and L. Wilson. 1977. Addition of colchicine-tubulin complex to microtubule ends: the mechanism of substoichiometric colchicine poisoning. *Proc. Natl. Acad. Sci. U. S. A.* 74:3466–3470.
26. Margolis, R. L., and L. Wilson. 1978. Opposite end assembly and disassembly of microtubules at steady state *in vitro. Cell.* 13:1–8.
27. Margolis, R. L., and L. Wilson. 1979. Regulation of the microtubule steady state *in vitro* by ATP. *Cell.* 18:673–679.
28. Nuccitelli, R., M. M. Poo, and L. R. Jaffe. 1977. Relations between ameboid movement and membrane-controlled electrical currents. *J. Gen. Physiol.* 69:743–763.
29. Olmsted, J. B., and G. Borisy. 1975. Ionic and nucleotide requirements for microtubule polymerization *in vitro. Biochemistry.* 14:2996–3004.
30. Oosawa, F., and S. Asakura. 1975. Thermodynamics of the Polymerization of Protein. Academic Press, Inc., London.
31. Schackmann, R. W., E. M. Eddy, and B. M. Shapiro. 1978. The acrosome reaction of *Strongylocentrotus purpuratus* sperm. Ion requirements and movements. *Dev. Biol.* 65:483–495.
32. Sloboda, R. D., W. L. Dentler, and J. L. Rosenbaum. 1976. Microtubule-associated proteins and the stimulation of tubulin assembly *in vitro. Biochemistry.* 15:4497–4505.
33. Steinhardt, R. A. 1978. Direct measurement of intracellular pH during metabolic derepression at fertilization and ammonia activation of the sea urchin egg. *Nature (Lond.).* 272: 253–354.
34. Tilney, L. G., D. P. Kiehart, C. Sardet, and M. Tilney. 1978. Polymerization of actin. IV. Role of Ca^{++} and H^{+} in the assembly of actin and in membrane fusion in the acrosomal reaction of echinoderm sperm. *J. Cell Biol.* 77:536–550.
35. Wegner, A. 1976. Head to tail polymerization of actin. *J. Mol. Biol.* 108:129–150.
36. Weisenberg, R. C. 1972. Microtubule formation *in vitro* in solutions containing low calcium concentrations. *Science (Wash. D.C.).* 177:1104–1105.
37. Wyman, J., Jr. 1964. Linked functions and reciprocal effects in hemoglobin: a second look. *Adv. Protein Chem.* 19:223–286.