Vol. 87, No. 4, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 27, 1979 Pages 1031-1038

EFFECTS OF SULFONATE ANIONS ON THE SELF-ASSEMBLY OF BRAIN TUBULIN

Richard H. Himes, Carol S. Newhouse, Kathryn M. Haskins, and Paul R. Burton

Department of Biochemistry, Department of Physiology and Cell Biology
University of Kansas
Lawrence, Kansas 66045

Received February 23, 1979

SUMMARY

Sulfonate buffers and aliphatic sulfonate salts influence the formation of microtubules in vitro in a manner that is not explained on the basis of ionic strength. Their presence stimulates assembly at higher pH values causing a broadening of the pH optimum curve. Assembly can occur in very high concentrations of sulfonate salts, at ionic strengths which inhibit assembly in the absence of these compounds. The effectiveness of the aliphatic sulfonate compounds is altered as the chain length is increased. Dimethylsulfoxide has an additional effect and influences the structure of the polymerized product.

INTRODUCTION

Solution conditions which support the self-assembly of tubulin in vitro have been described in a number of reports. Although such variables as pH, ionic strength, source of tubulin, and the presence or absence of organic solvents differ from laboratory to laboratory, an almost universal component of the assembly system is a zwitterionic sulfonate buffer. Usually morpholinoethanesulfonate (MES) or piperazinediethanesulfonate (PIPES) is used. In early studies such buffers were found to promote efficient assembly of brain tubulin (1, 2) and since then have been used routinely. Little documentation of the specific stimulatory effects of these sulfonic acid derivatives exists, however. In this article we present evidence which indicates that certain sulfonate compounds exert effects on the rate and extent of assembly and on the structure of the assembly product. These effects cannot be explained on the basis of ionic strength alone.

MATERIALS AND METHODS

Bovine brain tubulin was purified through two cycles of the assembly-disassembly method and stored in 2 M glycerol as described previously (3). Before the assembly reactions involving twice-cycled tubulin, glycerol was removed. The protein solution was thawed, ^{3}H-glycerol (5 μc/ml) and GTP (0.5

0006-291X/79/081031-08$01.00/0

mM) were added and the solution was incubated at 37° C for 20 min. The microtubules were collected by centrifugation, suspended in 20 mM PIPES, pH 6.9, containing 1 mM dithiothreitol, and dialyzed 1-3 hrs to remove remaining glycerol. Pure 6S tubulin was prepared by chromatographing twice-cycled protein on phosphocellulose (4).

In vitro assembly was monitored in a Gilford Model 2000 recording spectrophotometer. Reactions were done at 37° C in 0.5 ml containing 1 mM EGTA, 0.5 mM $MgCl_2$, 0.5 mM GTP and other components as described in the figure legends. Stock solutions (1 M) of methanesulfonic acid (Eastman), ethanesulfonic acid (Aldrich), 1-propanesulfonic acid (Eastman), sodium 1-butanesulfonate (Eastman) and sodium 1-heptanesulfonate (Regis) were made in 0.1 M PIPES and adjusted to pH 6.9 with NaOH. GTP, PIPES, HEPES, taurine, and EGTA were purchased from Sigma Chemical Co.

RESULTS

Effect of sulfonates and Me_2SO on the response of assembly to pH changes.

A number of laboratories have shown that tubulin self-assembly occurs best in the pH region 6.5 to 6.9. In general, it has been found that as the pH is increased to and above 7, the rate and the extent of polymerization decrease markedly. However, the steepness of this decrease depends on buffer conditions. For example, in our experience, with low concentrations (10-20 mM) of a sulfonate or non-sulfonate buffer like phosphate (with NaCl being added to maintain the ionic strength at about 0.1 μ) very little assembly is obtained at pH 7. More assembly is obtained at this pH with a 0.1 M sulfonate buffer. Such results are shown in Fig. 1. In this experiment the pH was maintained with a 20 mM buffer containing a mixture of MES and HEPES and the ionic strength was adjusted with either NaCl or ethanesulfonate. In the higher pH region, ethanesulfonate greatly stimulates assembly, whereas at lower pH values this stimulation is much less. Also shown is the effect of dimethylsulfoxide (Me_2SO) In both cases, assembly proceeds to an appreciable extent at higher pH values. In other experiments using 0.1 M HEPES, assembly was observed at pH values as high as 8.6. In all cases negative staining was used to confirm that microtubules were formed.

Effect of different sulfonate salts on assembly. In the course of doing the pH studies we found that a number of sulfonate compounds cause the stimulation of assembly at the higher pH values. These include the buffers MES, PIPES, TES,

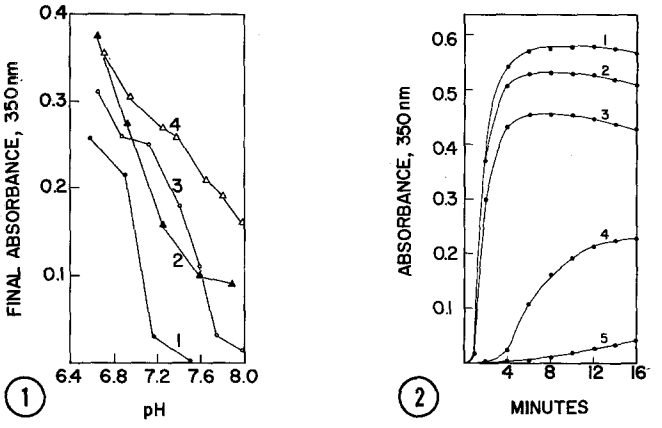

Fig. 1. Effect of ethanesulfonate and Me_2SO on assembly in the high pH region. Tubulin (1.9) mg/ml) was assembled at 37° C in an 0.5 ml volume in the presence of 20 mM MES-HEPES buffer, 1 mM EGTA, 0.5 mM $MgCl_2$ and 0.5 mM GTP. The buffers were made by mixing 1 M MES, pH 6.2, and 1 M HEPES, pH 8.86, in different proportions. The pH of each solution was measured after assembly was completed. Also included was either 0.1 M NaCl (Curve 1); 0.1 M NaCl and 10% Me_2SO (Curve 2); 0.1 M ethanesulfonate (Curve 3); or 0.1 M ethanesulfonate and 10% Me_2SO (Curve 4).

Fig. 2. Effect of different sulfonates on assembly. Assembly conditions were as described under "Materials and Methods" at pH 6.9 in the presence of 20 mM PIPES, 3.7 mg/ml protein, and 0.1 M of the following sulfonates; 1, ethane or methane; 2, propane; 3, butane; 4, none and 5, heptane.

and HEPES as well as ethanesulfonate. Since the buffer compounds have different pKa values and numbers of sulfonate groups, it is difficult to compare these compounds while maintaining constant sulfonate concentration and ionic strength. We therefore decided to compare some straight-chain aliphatic sulfonates in their ability to stimulate microtubule formation. The results using 0.1 M concentrations are shown in Fig. 2. Clearly methane- and ethanesulfonate produce the best stimulation. Propane- and butanesulfonate are almost as effective but heptanesulfonate lacks stimulatory activity. The latter compound actually is an inhibitor. For example, when 50 mM ethane- and 50 mM heptane-sulfonate were used together assembly was negligible when compared to 100 mM ethanesulfonate or 50 mM ethanesulfonate plus 50 mM NaCl. In other experiments it was found that taurine (2-aminoethanesulfonate) and the inorganic ions

sulfate, sulfite, and hydrogen sulfite were completely without stimulatory effect. The inorganic anions actually inhibited assembly at a 0.1 M concentration.

Effect of high concentrations of sulfonates. A number of investigators have shown that the self-assembly of tubulin has a narrow ionic strength optimum of about 0.1 μ. Concentrations of salts, including sulfonate buffers, giving higher ionic strength values inhibit microtubule formation. In most of these studies the salt concentrations were increased only to about 0.25 M. In our investigations we found that higher concentrations of sulfonate salts actually stimulated assembly, especially at higher protein concentrations (Fig. 3). At a tubulin concentration of 2.2 mg/ml, inhibition by 0.24 M ethanesulfonate is evident (Fig. 3A): however, by increasing the ethanesulfonate concentration further, the rate and extent of the assembly reaction then increase. Fig 3B shows the effect of protein concentration on the assembly in 0.64 M ethanesulfonate. Me_2SO has a large stimulatory effect at high sulfonate concentrations as shown in Fig. 3B.

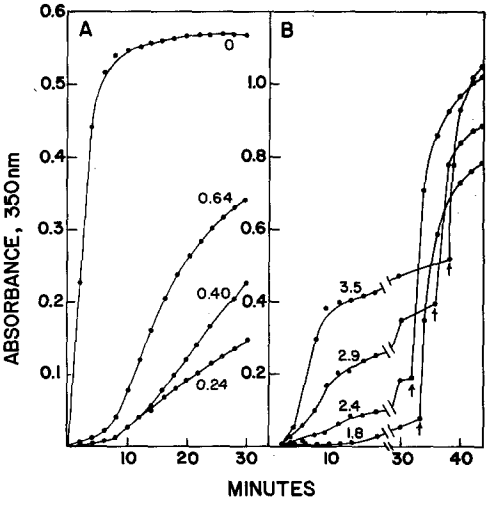

Fig. 3. Assembly at high sulfonate concentrations. Assembly conditions were as described under "Materials and Methods" except as noted. A. Tubulin, (2.2 mg/ml) and the ethanesulfonate concentrations (M) shown. PIPES, pH 6.9, was at 0.074 M. B. Tubulin (mg/ml) concentration varied as shown in 0.074 M PIPES, pH 6.9, and 0.64 M ethanesulfonate. At the arrows 50 ul Me_2SO was added to make the concentration 9%.

The various aliphatic sulfonates were tested at a 0.4 M concentration and compared to NaCl. The most effective compounds were the smallest, methanesulfonate and ethanesulfonate. The results using propane- and butanesulfonate were interesting. Although there was a slow increase in absorbance, the resulting turbidity did not decrease in the cold. Moreover, negative-stained samples showed the presence of aggregates, not microtubules. When the above experiments were done in the presence of 10% Me_2SO, large numbers of microtubules and wide ribbons of protofilaments were observed in the presence of methane-, ethane- and propanesulfonate. Aggregates were still observed with butanesulfonate and assembly was not observed with heptanesulfonate. In other experiments, assembly was achieved in 0.86 M PIPES with and without Me_2SO. In both cases examination by electron microscopy of fixed and sectioned material showed the presence of broad ribbons of protofilaments which had no filamentous material associated with them. Complete microtubules were not observed.

Effects of buffers and Me_2SO on the assembly of 6S tubulin. Tubulin purified by phosphocellulose chromatography to remove all traces of associated proteins can assemble at protein concentrations above 6 mg/ml (4) or at lower concentrations in the presence of additives such as Me_2SO (4), glycerol (5), a high Mg concentration (6), dextran (7), polyethyleneglycol (7), or α, β methylene GTP (8). We found that the sulfonate salts also affect this assembly process. For example, very poor assembly was obtained at pH 6.9 in the presence of 10% Me_2SO when the buffer was 20 mM phosphate, maleate, citrate, imidazole, or dimethylglutarate, supplemented with 0.1 M NaCl. At the same protein concentration, assembly was much better in 0.1 M PIPES. The assembly in the non-sulfonate buffers could be dramatically improved, however, if a sulfonate compound was added. Fig. 4A shows that assembly is stimulated by increasing the concentration of PIPES in a phosphate buffer, and also by further addition of Me_2SO to an imidazole buffer (Fig. 4B).

The assembly of 6S tubulin, like twice-cycled tubulin, was also stimulated by high concentrations of sulfonate compounds in the presence or absence of

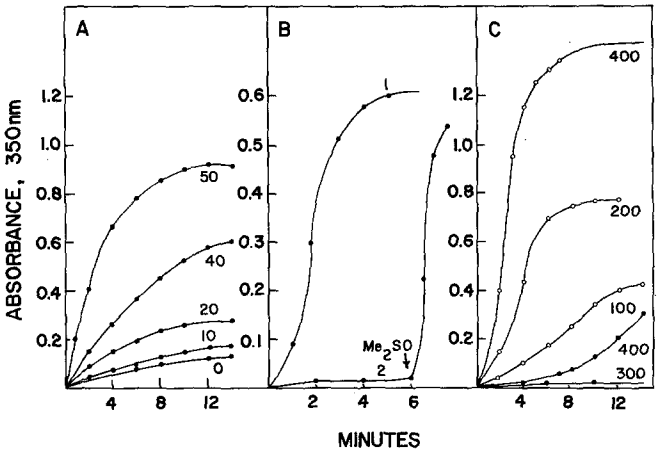

Fig. 4. Effect of sulfonates and Me_2SO on assembly of 6S tubulin. Assembly conditions were as described under "Materials and Methods" except as noted. A. Assembly of 2.2 mg/ml of 6S tubulin in 10 mM sodium phosphate, pH 6.9, 0.1 M NaCl, 10% Me_2SO and the concentration (mM) of PIPES, pH 6.9, shown. B. Assembly in 0.1 M PIPES, pH 6.9, 10% Me_2SO (Curve 1) and in 20 mM imidazole, pH 6.9, 0.1 M NaCl, 10% Me_2SO (Curve 2). Tubulin (6S) concentration; 1.5 mg/ml. At the arrow the Me_2SO concentration was increased to 20%. C. Assembly in various PIPES concentrations (mM) as shown. Tubulin (6S) concentration; 1.5 mg/ml. Open circles, in 10% Me_2SO; closed circles, no Me_2SO.

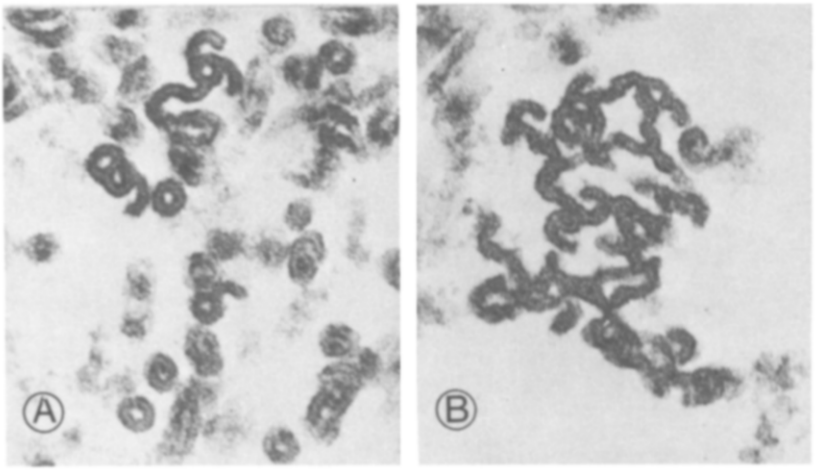

Fig. 5. Electron micrographs of 6S tubulin assembled in 0.4 M PIPES, pH 6.9. Samples were taken from the experiment described in Fig. 4C, fixed with glutaraldehyde, stained with tannic acid, and prepared for microscopy as described previously (10). Magnification, 210,000. A. Without Me_2SO. B. With 10% Me_2SO.

Me_2SO although with Me_2SO, much greater polymerization occurred. Some results using PIPES are shown in Fig. 4C. A significant difference was noted in the structure of the products assembled in 0.4 M PIPES depending on whether Me_2SO was present or absent. In 10% Me_2SO only complex ribbons were formed; however, when Me_2SO was not included complete microtubules were abundant. In the latter case other interesting forms such as doublet and triplet microtubules were observed. Shown in Fig. 5 are representative micrographs.

DISCUSSION

Organic sulfonates clearly stimulate tubulin assembly when compared to inorganic salts, especially above pH 6.5. Another property of the sulfonates is that they stimulate assembly at very high concentrations (the highest tested was 0.86 M PIPES). This characteristic has been reported for one non-sulfonate salt, potassium glutamate, which also supports assembly at concentrations up to 0.9 M (9). The assembly of twice-cycled tubulin under conditions of high sulfonate concentration actually involves polymerization of 6S tubulin. This results from the observation that at the high ionic strengths used, the binding of MAPs to tubulin is apparently weakened and rings and spirals present dissociate into 6S dimers (4).

The effect of sulfonates is fairly specific since increasing the carbon chain length greatly changes their action. There is little difference between methane- and ethanesulfonate, although propane- and butanesulfonate have unusual effects. At 0.1 M the latter two promote assembly. At 0.4 M they stimulate assembly of aggregates; on the other hand, microtubules are formed in 0.4 M propanesulfonate if Me_2SO is present. Heptanesulfonate actually inhibits assembly. These results suggest that there is a direct interaction of the sulfonate compound with the protein and the bulkier alkyl groups adversely affect tubulin-tubulin interactions.

The *in vitro* formation of microtubules depends on the interaction of a number of variables including pH, ionic strength, protein concentration, presence of organic solvents, and concentration of sulfonate anions. The

solution conditions not only affect the rate and extent of assembly but also the structure of the polymerized product. We (10) and others (11) have shown that the structure is affected by the pH of the solution. At the higher pH regions, complete microtubules are the predominant product; at lower pH values, complex ribbons are seen in high numbers. In our previous work (10), and in this report, we have shown that the solution conditions affect the relative proportions of ribbons and microtubules at any specific pH. In the assembly of 6S tubulin, for example, increasing sulfonate or Me_2SO concentrations extends the pH region over which ribbons or sheets are formed. The pH region where microtubules are formed is consequently also extended to higher values. Adding sulfonate and Me_2SO together magnifies this effect.

ACKNOWLEDGEMENT

This work was supported by NIH research grants NS 11360 (R.H.H.), Al 06448 (P.R.B.), GM 24992 (P.R.B.), American Cancer Society research grant CH-98 (R.H.H.) and a predoctoral traineeship to K.M.H. from Research Service Award HD 07066 to the Kansas Center for Mental Retardation and Human Development.

REFERENCES

1. Weisenberg, R. C. (1972) Science 177, 1104-1105.
2. Olmsted, J. B., and Borisy, G. G. (1975) Biochemistry 14, 2296-3005.
3. Lee, Y. C., Samson, F. E., Houston, L. L, and Himes, R. H. (1974) J. Neurobiol. 5, 317-330.
4. Himes, R. H., Burton, P. R., and Gaito, J. M. (1977) J. Biol. Chem. 252, 6222-6228.
5. Lee, J. C. and Timasheff, S. N. (1975) Biochemistry 14, 5183-5187.
6. Herzog, W., and Weber, K. (1977) Proc. Natl. Acad. Sci. 74, 1860-1864.
7. Herzog, W., and Weber, K. (1978) Eur. J. Biochem. 91, 249-254.
8. Sandoval, I. V., MacDonald, E., Jameson, J. L, and Cuatrecasas, P. (1977) Proc. Natl. Acad. Sci. 74, 4881-4885.
9. Suzaki, T., Sakai, H., Endo, S., Kimura, K., and Shigenaka, Y. (1978) J. Biochem. 84, 75-81. 10. Burton, P. R., and Himes, R. H. (1978) J. Cell Biol. 77, 120-133. 11. Matsumara, F., and Hayashi, M. (1976) Biochim Biophys. Acta 453, 162-175.