REGULATION OF MICROTUBULES IN *TETRAHYMENA*

II. Relation Between Turnover of Microtubule Proteins and Microtubule Dissociation and Assembly during Oral Replacement

NORMAN E. WILLIAMS and E. MARLO NEILSEN

From the Department of Zoology, University of Iowa, Iowa City, Iowa 52240

ABSTRACT

Experiments are reported which were designed to test for induced synthesis of microtubule proteins associated with the rapid proliferation of basal bodies and associated intracytoplasmic microtubules which occurs during oral replacement in *Tetrahymena*. None was found. Instead, it is shown that these structures can be formed with *de novo* synthesis of as little as 6% of their microtubule proteins. It is suggested that basal body proliferation may be controlled by synthesis of morphogenetic regulator proteins.

INTRODUCTION

The extensive dissolution and assembly of microtubule structures which occurs during oral replacement in *Tetrahymena* has been described at the ultrastructural level in the previous paper in this series (21). The present report is concerned with the relation between the synthesis of microtubule proteins and the differentiation of this microtubular complex during oral replacement in nongrowing cells.

*Tetrahymena*, like other cells both eucaryotic and procaryotic, undergoes turnover of cell proteins under conditions which do not permit net synthesis. The adaptive significance of this attribute is that it permits “biochemical differentiation” (Mandelstam [13]) in nongrowing cells, i.e., selective synthesis of certain required proteins at the expense of the amino acid content of other cell proteins. The main objective of the present study is to ask whether this type of induced synthesis of microtubule proteins occurs in association with differentiation of the microtubular structures of the oral apparatus (OA) in *Tetrahymena*.

The microtubular structures studied specifically in this investigation are the basal bodies of the OA and their interconnecting microtubules. Rannestad and Williams (15) have presented a method for isolating *Tetrahymena* OA as a fraction suitable for biochemical study, and have separated and identified microtubule proteins from this preparation. These methods have been used in the present study. As isolated, the OAs are devoid of cilia, and therefore the results pertain specifically to the microtubule proteins of basal bodies and associated intracytoplasmic microtubules.

The question has been approached by considering two extreme alternative hypotheses: (a) the OAs formed in nongrowing cells may be constructed entirely of newly synthesized microtubule proteins, or (b) they may be made exclusively from preexisting microtubule subunits. Intermediate situations are, of course, also possible. A resolution of these alternatives may help to clarify our understanding of the control mechanisms by which cells regulate microtubule assembly. It is known that protein synthesis is required for formation of OA microtubules in...
oral differentiation in tubules in flagella (18), neural processes (19), and microtubules in this system may be controlled by the supply of subunits. If, on the other hand, predominant use of preexisting subunits is found, without extensive induced synthesis of these proteins, then assembly is more likely controlled by synthesis of some type of protein which serves as a morphogenetic regulator.

The results indicate that there is no major induced synthesis of microtubule subunits during oral replacement in Tetrahymena. During synchronous oral replacement, which takes about 2 h, differentiation of the new microtubule structures makes use of about 94% preexisting subunits, the remainder being newly synthesized in accord with average turnover rates for Tetrahymena proteins of about 3%/h. It is considered that the synthetic requirement is probably not for microtubule subunit supply, but may reflect control of morphogenesis by the regulated synthesis of other proteins which promote assembly.

MATERIALS AND METHODS

T. pyriformis strain GL-C stock cultures were maintained in Frankel's medium (6) and grown for experiments in flask cultures of proteose-peptone supplemented with liver fraction I. (PPL medium) as described previously (15). The flask cultures containing 250 ml of medium were grown for 14 h to a density of about 1 X 10^6 cells/ml in the presence of either 2 or 4 µCi/ml of tritium-labeled protein hydrolysate (Schwarz Bio Research Inc., Orangeburg, N. Y.). The cells were then washed twice by gentle centrifugation and resuspended in 250-300 ml of Frankel's medium minus the amino acid source (tryptone). The cell density at this point was usually from 4-8 X 10^6 cells/ml.

Cell numbers were monitored from the time of the wash until the end of each experiment with the aid of a Coulter Counter (Coulter Electronics, Inc., Industrial Div., Hialeah, Fla.). Cell samples were withdrawn, prepared for light microscopy by the silver impregnation method, and scored for oral replacement as in the previous report in this series (21).

The cultures in most experiments were divided into two at 6 h after the wash (by which time cell division had stopped). One of these served as a control, while the other received an excess of unlabeled amino acids (chase). The chased culture, after addition of a concentrated solution of amino acids, contained all amino acids of the synthetic medium for Tetrahymena (20) except three, at a concentration ten times that of the synthetic medium. The three amino acids omitted were histidine, leucine, and tryptophane. In the experiments with synchronized cells, the synchronizing heat shock (34.0°-34.1°C inside the flask) was administered either for 7 or 13 h beginning immediately after addition of the amino acid chase.

The specific activity of total cell protein was determined from 5-ml samples taken at intervals beginning at the time the unlabeled amino acid chase was added to one of the cultures in each experiment. The protein was prepared by extracting the samples with hot perchloric acid. Protein concentration was determined by the Lowry et al. method (12), and radioactivity was determined by scintillation counting after solubilizing with NCS (Nuclear-Chicago Corp., Des Plaines, Ill.).

OAs were isolated by the method of Rannestad and Williams (15), modified slightly for smaller samples. The cells contained in 65-130 ml of culture were pelleted and lysed by the addition of 4 ml of 0.1% Triton X-100 (Sigma Chemical Co., St. Louis, Mo.) with rapid swirling. The lysate was combined with 15 ml of cold distilled water and put through a Logeman homogenizer (Logemann Bros. Co., Milwaukee, Wis.). A 1 M sucrose cushion was inserted into the bottom of a 50 ml centrifuge tube containing the lysate. The OAs were collected at the bottom of the cushion by centrifugation at 2,000 g for 10 min. The fraction was then prepared for electrophoretic separation of microtubule proteins by the method of Renaud et al. (16), except that dithiothreitol was substituted for mercaptoethanol and the sample was concentrated before electrophoresis by dialysis against 20% Carbowax. The preparation was included in a sample gel and subjected to electrophoresis using the general method of Davis (4). The polyacrylamide gels were made 8 M in urea and 7% in acrylamide. Electrophoresis was carried out until the bromophenol blue marker dye ran off the bottom of the gel. The gels were stained for 1 h with 1% fast green in 7% acetic acid, and destained in 7% acetic acid.

The two prominent bands nearest the anode in gels prepared from OA fractions (see Fig. 2) have previously been identified as microtubule proteins (15). Relative concentrations of these proteins were determined by cutting and weighing densitometric scans obtained with a Gilford microspectrophotometer with accessory gel carriage (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). The gels were then cut into successive 0.7 mm slices with a Joyce Loebel gel slicer (Joyce, Loebel and Co., Inc., Burlington, Mass.). The slices were put into scintillation vials and solubilized by shaking overnight in 0.1 ml of water and 0.5 ml of NCS solubilizer. Scintillation
fluid was then added and radioactivity was counted in a Beckman LS 133 scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). Specific activities are reported as counts per minute per gram paper weight of the gel scans. Preliminary tests showed that fast green stains protein in accordance with Beer’s law over the range used in the present study, and that specific activity determinations obtained by this method are reproducible to within a few percent.

RESULTS

Microtubule Protein Synthesis during Asynchronous Oral Replacement

The alternative hypotheses, the use of newly synthesized microtubule proteins for oral replacement vs. utilization of preexisting subunits, can be tested by determining what proportion of these proteins within the OA is synthesized during induced oral replacement. This was done by comparing specific activities of prelabeled OA microtubule proteins before oral replacement with specific activities of these proteins after oral replacement in the presence of an excess of unlabeled amino acids (chase). The effectiveness of the chase was determined by simultaneously measuring synthesis (turnover) of total cell protein. Ideally the chase should contain all amino acids; however, one or more essential amino acids must be omitted in order to prevent growth and ensure oral replacement. Three amino acids essential for Tetrahymena were withheld in the present experiments: histidine, leucine, and tryptophane. The requirement for withholding some amino acids from the chase has the consequence that the method will necessarily provide a slight underestimate of synthesis. Protein synthesis does occur under such conditions of starvation, however, as evidenced by the induction of enzymes in nongrowing cells (13).

An initial turnover rate of about 3%/h is indicated for total cell protein in the experiment presented in Fig. 1, thus demonstrating the effectiveness of the chase. Time zero is the time at which cells grown in the presence of 2 μCi/ml of tritium-labeled protein hydrolysate were washed twice and resuspended in amino acid-free medium. At 6 h, which was just after the culture switched from division to oral replacement (Fig. 1, top and bottom graphs), the culture was divided into two parts and the amino acid chase was given to one of these. There was a 46% decline in specific activity (Fig. 1, middle graph) in total cell protein in the chased culture over the interval from 6 h (beginning of the chase and time of first OA isolation) to 24 h (time of the second OA isolation). This indicates an average turnover rate for total cell protein of 2.6%/h over the experimental interval. It was confirmed in replicate experiments (not presented) that the decline in specific
activity, as measured with the present method, proceeds with decelerating kinetics (see also Fig. 3). Therefore the indicated turnover rate is initially somewhat higher and the final rate somewhat lower than 2.6%/h.

Cell division was stopped by 6 h (Fig. 1, top and bottom graphs), and oral replacement was found occurring in about 14% of the cells at all times in the population between 6 and 24 h (Fig. 1, bottom graph). It is estimated (see Discussion) that the average cell underwent from two to three oral replacements over this interval. Thus, a requirement for synthesis of significant quantities of OA microtubule proteins to be used in oral replacement would predict an extensive drop in the specific activities of the microtubule proteins within the OAs over the interval from 6 to 24 h in the chased culture. The extreme alternative hypothesis, exclusive use of preexisting microtubule subunits in oral replacement, would predict the absence of a decline in specific activities in OA microtubule proteins under these conditions.

OA fractions were therefore isolated from the chased culture at 6 and 24 h and the specific activities were determined (Table I) from microtubule proteins separated by polyacrylamide gel electrophoresis (Fig. 2). As seen in Table I, there was a decline in specific activity of microtubule protein of the OA of 35.5% over the experimental interval in one experiment, and a decline of 32.5% in a second. The decline rules out the exclusive use of preexisting subunits in oral replacement, whereas the fact that the decline in specific activity in OA microtubule proteins was only about 34% after two to three oral replacements per cell rules out induced specific synthesis of large quantities of OA microtubule protein. The decline in specific activity in the unknown OA proteins in the top of the gels was also determined in the first experiment in Table I, and it was found that the turnover of these proteins (54%) was similar to that of the average cell protein.

| Gel | Region | OD* | cpm | Sp Ac† | Decline |
|-----|--------|-----|-----|-------|---------|
| 6   | Tubulins | 0.54 | 926 | 1715 | -- |
|     | Top     | 1.76 | 3583 | 2036 | -- |
| 24  | Tubulins | 0.67 | 742 | 1107 | 35.5 |
|     | Top     | 2.23 | 2151 | 965 | 32.6 |
| 6   | Tubulins | 0.37 | 447 | 1208 | -- |
|     | Tubulins | 0.79 | 645 | 816 | 32.5 |

* Optical density is given as grams paper weight from densitometer scans.
† Specific activity is given as counts per minute per gram optical density.

**Figure 2.** Optical density and radioactivity profiles of OA proteins in polyacrylamide-urea gels. The proteins were prepared from OA fractions isolated at 6 and 24 h from the chased culture in the experiment presented in Fig. 1. The top curve for each sample is optical density, measured with a Gilford recording spectrophotometer with accessory gel carriage. The two microtubule proteins are the peaks in the center of each scan. The curves with points are the radioactivity profiles, determined by counting the radioactivity in successive 0.7 mm gel slices with a Beckman scintillation counter. Specific activities calculated from these data are presented in Table I, along with results of a duplicate experiment.
The results of the asynchronous oral replacement experiment suggest that microtubule protein of the OA turns over to amino acids at a rate slightly slower than the average cell protein during periods of prolonged asynchronous oral replacement, and that there is neither a mandatory conservation nor a major induced synthesis of microtubule subunits associated with this structural regression and redifferentiation.

**Microtubule Protein Synthesis during Synchronous Oral Replacement**

A more rigorous test for induced synthesis of microtubule protein is provided by experiments with cells undergoing synchronous oral replacement. In the synchronous system, virtually all cells undergo oral replacement in a short period of time (about 2 h), and therefore the decline in specific activity in OA microtubule protein in an experiment of the previous type will be slight if there is no induced synthesis. Conversely, the specific activity should drop extensively in a short period of time if induced synthesis occurs.

Oral replacement in *Tetrahymena* can be synchronized by the application of a prolonged heat shock to a culture undergoing asynchronous oral replacement. An experiment of this type is presented in Fig. 3. Zero time again is the time at which cells grown in the presence of 2 μCi/ml tritium-labeled amino acids were washed twice and suspended in amino acid-free medium. After cell division had stopped (arrow, Fig. 3) and oral replacement was occurring maximally (Fig. 3, bottom graph), the culture was divided into two. One half received the chase and both cultures were elevated to 34°C for 7 h. After the end of the synchronizing heat shock treatment (EST), a synchronous burst of oral replacement was observed in both the control and chased cultures (Fig. 3, bottom). Counts from silver preparations showed that 86% of the cells were in some stage of oral replacement in both the experimental and control cultures at EST plus 80 min. The bursts were completed by a little over 2 h after EST, with a few cells continuing oral replacement after this time.

The rate of turnover of total cell protein throughout the heat shock and the burst of synchronous oral replacement was very similar to that seen in unsynchronized cells. Fig. 3 (middle graph) shows that there was a specific activity decline of 26% in total cell protein over 9 h in the chased culture, or about 2.9%/h. Decelerating kinetics were again observed; therefore the initial rate indicated is slightly higher and the final rate slightly lower than 2.9%/h.

Turnover in the OA under these conditions was determined in two experiments presented in Table II. In the first experiment, OA fractions were isolated and specific activities of microtubule protein determined at the beginning of the heat shock.
TABLE II

| Sample | Quantity* | cpm | Sp Act | Decline % |
|--------|-----------|-----|--------|-----------|
| 6 h    | 830       | 1337 | 6 0.54 | 830       |
| 13     | 1276      | 1519 | 13 0.84| 1276      |
| 15     | 937       | 1420 | 15 0.66| 937       |

*Optical density is given as grams paper weight from densitometer scans.
† Specific activity is given as counts per minute per gram optical density.

It is seen from Table II that there was no decline in specific activity of OA microtubule protein during the heat shock. This interesting result is confirmed and considered further in the next section.

The test for induced synthesis of OA microtubule protein is the measurement of the decline in specific activity in the chased culture over the 2 h interval from 13 to 15 h, during which time virtually all of the cells resorbed existing OA structures and replaced them with newly differentiated structures. It is seen from the two experiments in Table II that the decline in specific activity of OA microtubule protein during the heat shock was only about 6%. This is not significantly different from the turnover which occurs in total cell protein in a 2 h period (see above), therefore no induced synthesis of OA microtubule protein associated with oral replacement is indicated. The fact that about 94% of the label present in the OA microtubules before oral replacement is also present after oral replacement in chase medium suggests that any induced synthesis of this type of protein is no more than a few percent. Clearly, the majority of the subunits of microtubules involved in this differentiation need not be synthesized during development.

The Effect of Synchronizing Heat Shocks on Turnover

The previous experiment indicates that the synchronizing heat shocks blocked turnover of microtubule protein in the OA but did not block turnover in total cell protein. This conclusion is confirmed by the additional experiments presented in Table III. The latter experiments were done in exactly the same way as the experiment presented in Fig. 3 and Table II, except that the heat shock (34°C) was administered for 13 h rather than for 7 h, and specific activities were determined in total cell and OA microtubule protein only at the beginning (6 h) and the end (19 h) of the extended heat shock. The longer heat shock was used in order to allow for more extensive turnover, should this process be occurring at 34°C.

The results in Table III show that there was a decline in specific activity of total cell protein of 31.4% during 13 h at 34°C, and (in two separate experiments) again no decline in specific activity of OA microtubule protein was detected. Two possible interpretations of this result may be considered. Either turnover of microtubule protein does not occur in OAs which are not undergoing oral replacement (the latter process is prevented

TABLE III

| Sample         | Quantity* | cpm  | Sp Act | Decline % |
|----------------|-----------|------|--------|-----------|
| 6 h total cell | 78        | 68,731| 881    | 6 0.684   |
| 19 h total cell| 79.5      | 48,045| 604    | 31.4      |

* Cell protein in micrograms, microtubule proteins in grams paper weight from densitometer scans.
† Specific activity of total cell protein is given as counts per minute per microgram protein; microtubule protein specific activity is counts per minute per gram paper weight from densitometer scans.

Norman F. Williams and E. Marlo Nelson Turnover of Microtubule Protein, II 463
DISCUSSION

A major conclusion from the present study is that there is apparently no induced synthesis of oral microtubule protein associated with oral replacement in *Tetrahymena*. The microtubule structures of this extensive system of interconnected basal bodies can form synchronously in a population within 2 h under conditions in which it is demonstrated that only about 6% of the microtubule protein in the structures is newly synthesized (Fig. 3, Table II). The bulk of the microtubule subunits must preexist in the cells, and may include the subunits of the resorbed OAs, used again in the formation of the new structures.

This is consistent with, but not proven by, the results of the previous electron microscope study, which show that resorption of the old OA during oral replacement involves a loss of microtubular structure without involvement of autophagic vacuoles (21). Evidence for reutilization of flagellar proteins in regeneration of *Chlamydomonas* flagella has been reported by Coyne and Rosenbaum (3).

The absence of induced synthesis of microtubule protein in oral replacement is also indicated by the results of the more indirect experiment with asynchronous cells (Fig. 1, Table I). The interpretation of the decline in OA microtubule specific activity in relation to the possibility of induced synthesis in this experiment requires an estimate of the amount of oral replacement occurring over the interval measured. The previous study by Frankel (8) provides the basis for this estimate. Frankel concluded that oral replacement in *T. pyriformis* in amino acid-free medium is cyclic with a rather irregular period. A rough, though reasonable, estimate of the average period from Frankel's data is about 6-9 h. The 18 h interval from 6 to 24 h in Fig. 1 may therefore be considered as a period in which the cells had switched from cyclic divisions to cyclic oral replacement, and during which the average cell underwent from two to three oral replacement events. Table I shows that the OA microtubule protein specific activity dropped only about 33% under chase conditions over this interval of extensive structural regression and reformation, therefore extensive use was made of preexisting subunits and no major induced synthesis is indicated.

It has been previously shown that protein synthesis is an absolute requirement for oral replacement in *T. pyriformis* (8), as well as for the development of division oral primordia in this species (7, 9). Protein synthesis has also been found to be required for the formation of microtubules in a wide variety of systems, including regenerating *Stentor* oral primordia (2, 10), regenerating cilia in *Tetrahymena* (17), regenerating flagella in *Chlamydomonas* (18), and developing neural processes (19). This is apparently not true for all microtubule systems, however, because development of microtubules in the absence of protein synthesis has been reported in sea urchin embryos (1). The present finding that oral development in *Tetrahymena* may involve utilization of preexisting microtubule protein subunits to an extent of about 94% of the total found in the structure shows that the de novo synthesis requirement in this system is not for the major supply of subunit proteins. The required synthesis, therefore, may well be for a "regulator protein," i.e., a protein of one type which controls morphogenesis of structures made up largely of proteins of another type. Interactions of this type are well documented in studies of bacteriophage T4 morphogenesis (11, 23). Regulator proteins in *Tetrahymena* OA development may conceivably include microtubule proteins. Originally, microtubules were believed to be composed of a single protein. Later, better separations showed two species of microtubule proteins, and a recent report established the existence of three and perhaps more species of microtubule proteins (14). Therefore, measurements which are adequate to show that formation of microtubules does not involve induced synthesis of 94% of the microtubule protein subunits cannot exclude the possibility of morphogenetic regulation by induced synthesis of a small number of subunits of a particular type. Other possibilities include regulation of development by controlled synthesis of structural proteins other than the tubulins, or of proteins which function catalytically to promote assembly. Whatever the nature of the required synthesis, it appears not to be for bulk subunit supply.

The method for measuring turnover of protein in the present study, based upon dilution of prelabeled protein by unlabeled amino acids, gives an underestimate because of the necessity of
withholding some amino acids from the chase to insure the occurrence of oral replacement and prevent growth. However, turnover rates of 1%/h are reported for mammalian cells in culture (5) and 5%/h for starved *Escherichia coli* (13), therefore the present estimates of protein turnover in *Tetrahymena* at about 3%/h appear reasonable.

Turnover measurements in *Tetrahymena* during the synchronizing heat shock (34°C) revealed a significant difference between total cell protein and microtubule protein within the OA in their responses to the elevated temperature. Whereas the turnover rate in total cell protein at 34°C was slightly higher than the turnover rate at optimum growth temperature (28°C), no turnover at all was detected in OA microtubule protein during oral replacement, which it does, thereby maintaining all OAs in a morphostatic condition. The following argument can be made for the first interpretation. It is known from the results of the experiment with synchronous oral replacement that the process takes about 2 h during which there is an approximately 6% turnover in OA microtubule protein. It was also estimated that the number of oral replacements occurring over 18 h in the asynchronous experiment was two to three per cell. Therefore, if turnover of OA microtubule protein occurred only during oral replacement, and further, if the rate of synthesis of microtubule protein during oral replacement is the same in synchronized and unsynchronized cells, then the decline in specific activity of this protein in the asynchronous cells should have been 6% times two to three oral replacement events, or from 12–18%. The data (Table I) shows a decline in specific activity of about 33%, or close to twice that to be expected on the basis of oral replacement alone. Therefore, the OAs appear to undergo turnover of microtubule proteins when they are morphostatic as well as when they are morphogenetic. The absence of turnover during heat then suggests that this process is heat sensitive. This is consistent with the results of a previous autoradiographic study in which it was concluded that proteins in mature oral structures of *Tetra-

*REFERENCES*

1. *Aucclair,* W., and B. W. *Siegel.* 1966. Science (Wash. D.C.) 154:913.
2. *Burchill,* B. R. 1968. *J. Exp. Zool.* 167:427.
3. *Coyne,* B., and J. L. *Rosenbaum.* 1970. *J. Cell Biol.* 47:777.
4. *Davis,* B. J. 1964. *Ann. N. Y. Acad. Sci.* 121:404.
5. *Eagle,* H., K. A. *Piez,* R. *Fleischman,* and V. A. *Oyama.* 1959. *J. Biol. Chem.* 234:592.
6. *Frankel,* J. 1965. *J. Exp. Zool.* 159:113.
7. *Frankel,* J. 1969. *J. Cell Physiol.* 74:135.
8. *Frankel,* J. 1970. *J. Exp. Zool.* 175:79.
9. *Gavin,* R. H., and J. *Frankel.* 1969. *J. Cell Physiol.* 74:123.
10. *James,* E. A. 1967. *Dev. Biol.* 16:577.
11. *King,* J. 1968. *J. Med. Biol.* 32:231.
12. *Lowry,* O. H., N. J. *Rosebrough,* and A. L. *Farr.* 1951. *J. Biol. Chem.* 193:265.
13. *Mandelstam,* J. 1960. *Bacteriol. Rev.* 24:289.
14. *Olmsted,* J. B., G. B. *Witman,* K. *Carlson,* and J. L. *Rosenbaum.* 1971. *Proc. Natl. Acad. Sci.* 68:2273.
15. *Rannestad,* J., and N. E. *Williams.* 1971. *J. Cell Biol.* 50:709.
16. *Renaud,* F. L., A. J. *Rowe,* and I. R. *Gibbons.* 1968. *J. Cell Biol.* 36:79.
17. *Rosenbaum,* J. L., and K. *Carlson.* 1969. *J. Cell Biol.* 40:415.
18. *Rosenbaum,* J. L., J. E. *Moulde,* and D. L. *Ringo.* 1969. *J. Cell Biol.* 41:600.
19. *Schubert,* D., S. *Humphreys,* F. *DeVitry,* and F. *Jacob.* 1971. *Dev. Biol.* 25:514.
20. *Shaw,* R. F., and N. E. *Williams.* 1963. *J. Protozool.* 10:496.
21. *Williams,* N. E., and J. *Frankel.* 1973. *J. Cell Biol.* 56:441.
22. *Williams,* N. E., O. *Michelsen,* and E. *Zeuthen.* 1969. *J. Cell Sci.* 5:143.
23. *Wood,* W. B., and M. *Henning.* 1969. *J. Med. Biol.* 39:603.

*Norman E. Williams and E. Maibo Nilsen* Turnover of Microtubule Protein. II 465