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Programmed Synthesis of Tubulin for the Flagella that Develop During Cell Differentiation in *Naegleria gruberi*

(microtubules/isotope dilution/gel electrophoresis/radioimmune assay)

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ABSTRACT **Amebae of *Naegleria gruberi* differentiate into flagellates when transferred from growth medium to nonnutrient buffer. Experiments were performed to determine whether the tubulin that forms the flagellar microtubules pre-exists in amebae or is synthesized during differentiation. Amebae prelabeled uniformly with [^{35}S]-methionine were allowed to differentiate in the presence and in the absence of exogenous unlabeled methionine. In the presence of unlabeled methionine the flagellar tubulin contained only 30% as much [^{35}S]methionine as in its absence. Thus at least 70% of the tubulin was synthesized *de novo*. Isotope dilution and pulse experiments showed that flagellar tubulin synthesis began one-third of the way through differentiation, before any morphological change had occurred. Flagellar tubulin antigen, as measured using a specific antiserum, also began to increase one-third of the way through differentiation and increased 35- to 55-fold during the course of differentiation. These experiments demonstrate that most if not all of the flagellar tubulin is synthesized *de novo* during differentiation.**

The morphological and biochemical similarities between microtubules from flagella, the mitotic apparatus, and the cytoplasm suggest the possibility that all microtubules of a cell may be built from a common pool of tubulin subunits. Although tubulins from different microtubules are not identical (1–4), the possibility of interconversion between the various forms has not been eliminated. Inoué's dynamic equilibrium model of mitosis (5) suggested the existence of a cytoplasmic pool of tubulin in equilibrium with polymerizing mitotic microtubules, and Rosenbaum's studies of flagellar regeneration have indicated the existence of a pool of re-usable tubulin in equilibrium with flagellar microtubules (6). Since some organisms resorb their flagella during mitosis (7), it is possible that tubulin for both flagella and mitotic microtubules are drawn from a common pool.

We have analyzed the origin of flagellar tubulin during the differentiation of amebae to flagellates in *Naegleria gruberi*. Amebae transferred from growth medium to nonnutrient buffer differentiate rapidly and synchronously into transient, nonreproducing flagellates (8). This differentiation is dependent on both transcription and translation (9). A cell forms two 15-μm flagella, each with 20 tubules, whereas the spindle of a mitotic ameba contains more than 200 microtubules, each also about 15 μm long (C. Fulton, unpublished observations). Therefore, an ameba, at least at the time of mitosis, contains more than enough "tubulin" to provide for flagella. We have performed experiments to determine whether the cells use mitotic tubulins, or any preexisting tubulins, for flagellum construction.

Outer doublet tubulin of *Naegleria* flagella has been purified and characterized, and a radioimmune assay has been developed for this protein (10). Using this assay, we have shown that there is a 35- to 55-fold increase in flagellar tubulin antigen during differentiation (10). This increase may be due either to a change in antigenicity of preexisting tubulin subunits which are then used for flagellar construction, or to the *de novo* synthesis of flagellar tubulin†. Experiments presented here show that the latter is the case: most, and probably all, of flagellar tubulin is synthesized from amino acids during differentiation, and the time of appearance of tubulin antigen corresponds to the time of synthesis of tubulin polypeptide.

MATERIALS AND METHODS

Growth of Axenic Amebae and Differentiation to Flagellates. Amebae of *Naegleria gruberi*, strain NEG-M, were grown in suspension in Medium M at 32° (11). To initiate differentiation, amebae were centrifuged (Method C, ref. 8) and resuspended in TK buffer (2 mM Tris·HCl, 10 mM KCl, pH 7.2) at time zero. After several washes, the amebae were suspended in TK and shaken at 25°. Samples fixed at intervals in Lugol's iodine were counted for percentage of cells with flagella (12). Cells grown axenically have a T_{50} (time for 50% of the cells to become flagellates) of about 80 min and 80–95% have differentiated by 120 min (11).

Purification of Tubulin. The methods used here are based on those described fully elsewhere (10). When flagellates had full-length flagella, they were sedimented, resuspended in ice-cold acetate buffer (0.15 M sucrose, 2 mM $MgCl_2$, 10 μM ethylene diaminetetraacetate, 10 mM sodium acetate, pH 3.7

Abbreviation: SDS, sodium dodecyl sulfate; TK, a Tris–potassium buffer.

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† Flagellar tubulin refers to the tubulin in an acetone extract of Sarkosyl-treated flagella. Most if not all of this tubulin comes from intact outer-doublet microtubules. This tubulin has the molecular heterogeneity found in tubulins of other species and forms a doublet band when electrophoresed on urea gels. At present this heterogeneity among tubulins is not well defined in any system. In the experiments described in this paper the tubulin behaves as a single species.

at 20°), vortexed 5 sec to detach flagella, and immediately brought to pH 8. Cell bodies were removed by low-speed centrifugations. All subsequent steps were performed at 0–4°. The flagella were sedimented, resuspended in TDEG (25 mM Tris·HCl, 0.1 mM dithiothreitol, 0.1 mM ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid, 0.1 mM GTP, pH 7.6). Two volumes of a buffer with Sarkosyl detergent were added to disrupt membranes [38 mM Tris·HCl, 0.3 mM ethylenediaminetetraacetate, 0.075% (w/v) Sarkosyl (Geigy Industrial Chemicals), pH 7.6]. After 30 min the microtubule material was sedimented and washed once in TDEG by centrifugation. The final pellet was extracted twice with cold (−5°) acetone. The dried acetone powder was suspended in 0.1 ml of 1 mM Tris·HCl with 0.1 mM GTP, pH 7.6, centrifuged 15 min at 27,000 × *g*, and the supernatant, containing tubulin, was stored at −20°.

Labeling Cells. [^{35}S]Methionine (16–35 Ci/mmol, Amersham-Searle) was sterilized by passage through a Millipore HA filter. To label differentiating cells, isotope was dried at room temperature under a stream of nitrogen (to remove mercaptoethanol, which otherwise inhibits differentiation) and redissolved in TK. Measurement of radioactivity in trichloroacetic acid-precipitated total cell protein was done as described (9).

Electrophoresis. Methods for electrophoresis of tubulin on sodium dodecyl sulfate (SDS)-polyacrylamide gels and on pH 8.9 gels with or without 8 M urea have been described (10). Gels were stained 2 hr in Fast Green FCF (13), destained in 7.5% (v/v) acetic acid by diffusion, and were scanned with a Joyce–Loebl MK III C microdensitometer with a red filter. For measuring radioactivity, 1-mm slices were dissolved in 0.25 ml of 30% H_2O_2 for 5 hr at 60°. Three milliliters of Aquasol (New England Nuclear Corp.) was added after cooling, and the radioactivity measured in a Packard Tri-carb scintillation counter.

Immunoassay. The preparation of rabbit antiserum against flagellar tubulin, the preparation of cell extracts, and the radioimmune assay have been described (10). Extracts were made from cells of strain NEG that were grown in association with *Aerobacter aerogenes* (8) and differentiated at 28° in Tris buffer (2 mM Tris·HCl, pH 7.2) (12). Whole cell extracts were prepared from samples at various stages of differentiation by sonication and $(NH_4)_2SO_4$ precipitation of tubulin. The radioimmune assay measures flagellar tubulin antigen by measuring competition, for binding to antitubulin antibody, between extract and a standard amount of ^{125}I-labeled flagellar tubulin. Since the assay is based on competition for binding of electrophoretically purified flagellar tubulin, only tubulin is measured (10).

RESULTS

De novo synthesis of tubulin

The synthesis of at least some flagellar tubulin during differentiation of amebae to flagellates is shown by simple incorporation experiments. When cells differentiate in the presence of a radioactive amino acid, and the flagellar tubulin is electrophoresed on either SDS or urea gels, the tubulin is radioactive. Such experiments do not reveal what proportion of the flagellar tubulin is synthesized *de novo*.

A quantitative estimate of the fraction of tubulin synthesized can be obtained using prelabeled amebae. Amebae are grown for several generations in [^{35}S]methionine to uniformly label all protein and amino-acid pools. Since differentiation occurs in nonnutrient buffer, the only source of methionine for protein synthesis is the endogenous radioactive amino acid. However, if prelabeled cells are provided with exogenous unlabeled methionine, and if the cells use this methionine for protein synthesis, then newly synthesized proteins will contain less radioactivity than preexisting proteins. This approach allows an estimate of the extent of *de novo* synthesis by comparing the specific activity of flagellar tubulin in prelabeled cells that differentiated under two conditions: (*1*) in the presence of unlabeled exogenous methionine ("chased") versus (*2*) in buffer alone (control). Such an experiment gives a precise measure of the extent of *de novo* synthesis only if the chase is 100% effective, i.e., if newly synthesized proteins contain exclusively unlabeled methionine. Otherwise the experiment gives a minimal estimate. Since the utilization of exogenous methionine for protein synthesis increases proportionally with concentration (unpublished observations), we used the highest concentration for the chase that would permit differentiation without delay, 50 mM.

The isotope dilution experiment was performed with amebae prelabeled with [^{35}S]methionine during axenic growth for 5 generations. The amebae were washed into TK buffer to initiate differentiation. One half of the sample was suspended in TK (control) and the other half in TK containing 50 mM L-methionine (chased). When the cells had full-length flagella, at 135 min, the flagella were removed, and flagellar tubulin was prepared and electrophoresed on SDS gels. The absorbance scans of the stained gels from the two samples are almost superimposible (Fig. 1), indicating similar recovery of tubulin from samples with the same number of cells, treated in parallel. However, the specific activity of flagellar tubulin from cells that differentiated in the presence of methionine was only 38% of that of the tubulin from the control cells.

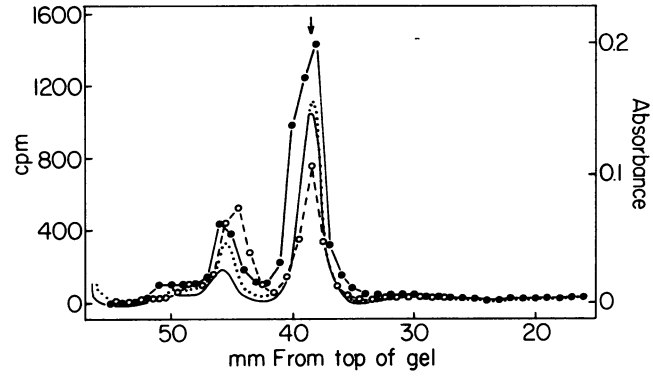

FIG. 1. *De novo* synthesis of tubulin. Amebae were prelabeled by growth in Medium M containing [^{35}S]methionine at 8.5 μCi/ml for 48 hr. The amebae were washed into TK buffer. One half (control) were allowed to differentiate in TK, the other half (chased), in TK containing 50 mM L-methionine. After 135 min at 25°, flagellar tubulin was prepared and electrophoresed on SDS gels. Gels were stained with fast green, scanned for absorbance, and then sliced for measurement of radioactivity. The *arrow* indicates the position of tubulin based on a parallel gel with pure tubulin. Control: absorbance ——, radioactivity ●——●. Chased: absorbance · · · ·, radioactivity O– – –O. No absorbance or radioactivity was found in the first 20 mm of the gels. The pooled tubulin fractions had 4530 cpm in the control and 1740 in the chased sample. The fast-moving protein, running ahead of tubulin, had 1690 and 1830 cpm, respectively.

TABLE 1. *Radioactive amino acids in [^{35}S]methionine-labeled tubulins*

Sample chromatographed	R_F	cpm
"Control" tubulin hydrolysate		
Methionine sulfone	0.22	382 (85%)
Cysteic acid	0.08	71 (15%)
"Chased" tubulin hydrolysate		
Methionine sulfone	0.22	125 (70%)
Cysteic acid	0.08	52 (30%)

Samples of the control and chased tubulin samples from the experiment of Fig. 1 were electrophoresed on SDS gels. Unstained 1-mm gel slices were eluted in 0.1% SDS. The radioactive tubulin peaks were localized and pooled, and 100 μg of carrier bovine-serum albumin was added. Protein was precipitated with cold acetone (14), washed with acetone to remove SDS, and lyophilized. This protein was oxidized with performic acid (15) and acid hydrolyzed in 5.5 M HCl *in vacuo* at 100° for 24 hr. Dried samples were dissolved in a solution containing 20 mM each of methionine sulfone and cysteic acid, and an aliquot of this mixture applied to Whatman no. 1 paper for ascending chromatography at room temperature in 1-butanol:glacial acetic acid:water (80:20:34). After 5 hr the paper was dried and stained with ninhydrin. The radioactivity in the two ninhydrin-positive spots was measured. No radioactivity was found other than in the two ninhydrin spots.

(In a separate experiment, the specific activity of the chased tubulin was 33% that of the control.) The decrease in specific activity of the tubulin from the chased sample must have been due to incorporation of exogenous unlabeled methionine into newly synthesized tubulin protein.

Amebae prelabeled with [^{35}S]methionine during growth contained a significant amount of label present as [^{35}S]-cysteine. Since *Naegleria* flagellar tubulin contains one-third of its sulfur in cysteine (10), and since only methionine was used for the chase, it was important to compare the relative specific activity of the methionine in the tubulins. Samples of tubulin from the previous experiment were hydrolyzed to amino acids, and the amino acids were separated by chromatography and their radioactivity was determined (Table 1). Of the total counts in the tubulin peaks of Fig. 1, 85% of the control counts but only 70% of the chased counts were recovered in methionine. When the total counts were corrected to include only the radioactivity in methionine, the chased tubulin was found to contain 30–31% of the methionine radioactivity in control tubulin. Thus 69–70% of the methionine radioactivity in tubulin was chased during differentiation. This is thus a minimal estimate of the extent of *de novo* synthesis of flagellar tubulin.

The fast-moving protein of Fig. 1 is the only other major protein species in these tubulin preparations. It is not a tubulin component, and can be eliminated by further purification of outer doublets (10). The fast-moving material is not appreciably synthesized during differentiation, as shown by the absence of a decrease in relative specific activity during the chase (Fig. 1) and by the lack of incorporation of label present during differentiation (see below).

Timing of tubulin synthesis

Is flagellar tubulin synthesized continuously during differentiation or does synthesis begin at a specific time? Two

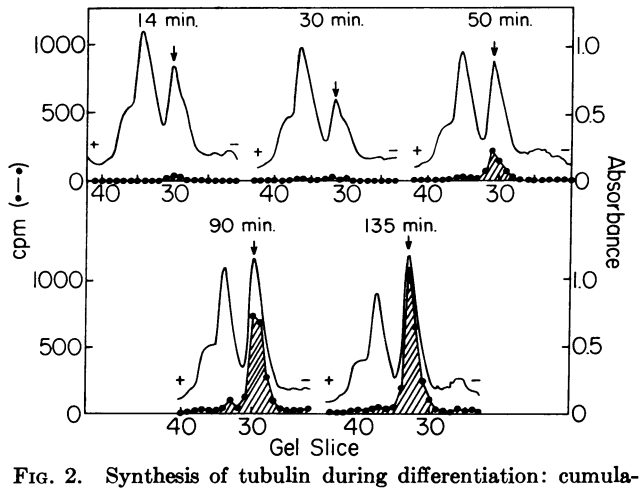

FIG. 2. Synthesis of tubulin during differentiation: cumulative labeling. Axenically grown amebae were washed and, at 14 min, divided into five flasks, each containing 3 ml of TK buffer with 8 × 10^7 cells and 72 μCi of [^{35}S]methionine. At various times, indicated on the graph, 1 ml of 0.2 M L-methionine was added to each flask to reduce further incorporation of label. At 135 min, when more than 90% of the cells had differentiated to flagellates, flagellar tubulin was prepared from each sample and electrophoresed on SDS gels. The gels were stained and scanned, then sliced, and the radioactivity measured. The *arrows* indicate the tubulin band. The fast-moving material was not appreciably labeled.

different approaches were taken to this question. In the first, aliquots of cells differentiating in [^{35}S]methionine were added at various times to an excess of unlabeled methionine to reduce further incorporation. After cells from all aliquots had completed differentiation, flagella were removed and the tubulins analyzed by electrophoresis. If samples were diluted before the initiation of tubulin synthesis, the flagellar tubulin in such samples should remain relatively unlabeled. There was no increase in radioactivity above the low amount incorporated after dilution unless samples were kept in [^{35}S]methionine for more than 30 min (Fig. 2); after 30 min the specific activity of the tubulin increased with labeling time. A plot of the specific activity of the tubulin versus time indicates the time course of cumulative tubulin synthesis during differentiation (Fig. 3). The failure to find incorporation, and therefore synthesis, in the first 30 min is not due to a change in permeability or in the rate of total protein synthesis, since incorpora-

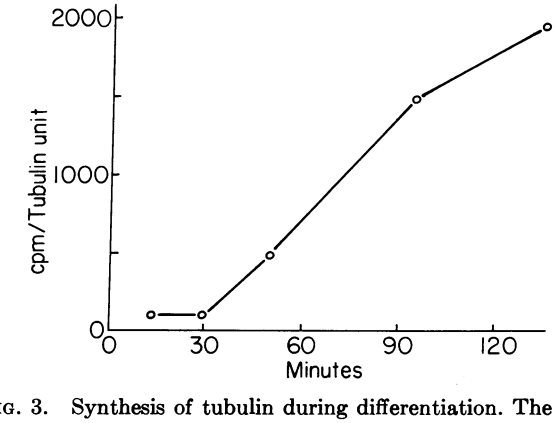

FIG. 3. Synthesis of tubulin during differentiation. The relative specific activities were determined from the data shown in Fig. 2.

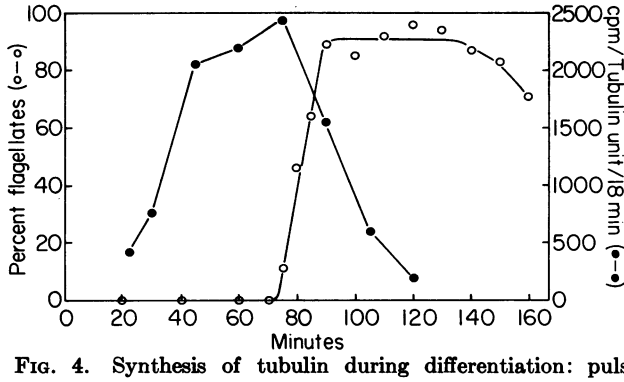

FIG. 4. Synthesis of tubulin during differentiation: pulse labeling. Part of this experiment is described in Table 2. Axenically-grown amebae were allowed to differentiate at 25°. The time course of appearance of cells with flagella is shown. Samples of the cells were exposed to [^{35}S]methionine for 18 min and then allowed to continue differentiation in 50 mM L-methionine. The flagella of these pulse-chased cells were isolated at 135 min and the specific activity of the tubulin measured. The rate of tubulin synthesis is plotted as a function of the time each pulse ended.

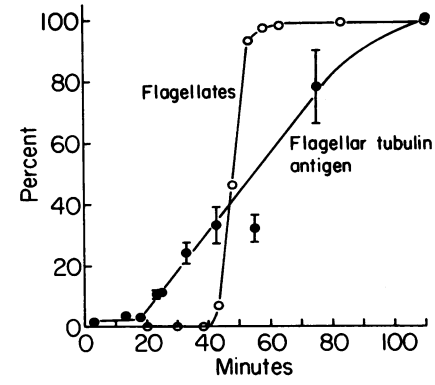

FIG. 5. Appearance of flagellar tubulin antigen during differentiation. Bacteria-grown cells were incubated in Tris buffer at 28°. Extracts prepared from cells taken at different times during differentiation were assayed for tubulin antigen by the radioimmune assay. The amount of antigen present at 110 min was defined as 100%. The *error bars* represent the range of values from duplicate assays of the extracts.

tion of [^{35}S]methionine into total cell protein commences immediately after addition of isotope (observed in this experiment; see also Table 2 and ref. 9). Synthesis of flagellar tubulin therefore begins 30–50 min after initiation of differentiation.

The rate of tubulin synthesis during differentiation was estimated by a different experiment. Aliquots of cells were pulse labeled with [^{35}S]methionine at various times during differentiation. After each 18-min pulse the cells were diluted to 50 mM nonradioactive L-methionine and allowed to complete differentiation. Samples were taken during each pulse to determine the amount of [^{35}S]methionine incorporated into total cell protein and at the end of differentiation to determine how much further incorporation took place after the pulse. The rate of incorporation of radioactive methionine into total cell protein was highest during the first half of differentiation, and finally decreased to about half the initial rate (Table 2, column 2). The results of total incorporation verify that both pulse and chase were effective throughout differentiation.

TABLE 2. *Incorporation of [^{35}S]methionine into total protein and into flagellar tubulin versus time during differentiation*

Time of pulse (min)	Incorporation into total protein (cpm/mg of protein × 10^{-3})		Percent flagellates at 120 min	Specific activity of flagellar tubulin*
	During pulse	During chase (at 120 min)		
4–22	118	−1	67	410
12–30	79	25	54	763
27–45	95	27	93	2050
42–60	101	0	92	2190
57–75	77	2	92	2440
72–90	55	13	95	1550
87–105	50	8	89	602
102–120	41	—	93	198

Eight 3-ml aliquots each containing 8×10^7 axenically grown cells were allowed to differentiate. At intervals, 0.66 mCi of [^{35}S]-methionine in 1 ml of buffer was added to a flask. Eighteen minutes later the contents of the flask were poured gently into 12 ml of TK containing L-methionine to give a final concentration of 50 mM. Samples were taken and precipitated with trichloroacetic acid to determine radioactivity in total cell protein just after addition of isotope, just before dilution, and at 120 min. At 120 min, samples were also taken to determine the percentage of cells with flagella. At 135 min the flagella were isolated, tubulin was prepared and separated by electrophoresis, and the relative specific activity was measured.

* cpm/unit of absorbance in tubulin peak per 18-min labeling.

When each aliquot of pulse-chased cells completed differentiation, the flagella were removed and their tubulin separated on SDS gels, and the specific activity of the tubulin was determined. The rate of synthesis of tubulin began to increase at 30 min, well before flagella first appeared, and reached a plateau level which was maintained until 75 min (Table 2 and Fig. 4). The drop after 75 min may reflect the assembly of tubulin subunits into flagella rather than a decline in rate of tubulin synthesis, since assembled subunits would be removed from the pool of subunits that could become labeled during a pulse (25).

These experiments indicate that tubulin is not synthesized throughout differentiation, but instead synthesis begins at about 30 min after initiation of differentiation, reaches a maximal level, and maintains that level at least until assembly of flagella is underway.

Synthesis of tubulin antigen

At least 70% of the tubulin polypeptide is synthesized during differentiation, and synthesis begins at a particular time. In a previous study, using a radioimmune assay for flagellar tubulin, we found that 97–98% of the flagellar tubulin antigen appears during differentiation (10). We used the radioimmune assay to determine the time-course of appearance of tubulin antigen during differentiation, and to see whether the time of antigen appearance is correlated with the time of synthesis of tubulin polypeptides.

Large quantities of cells are required for the radioimmune assay, so amebae grown on bacteria were used. These bacteria-grown cells differentiate more rapidly than cells grown on the axenic medium (9). The T_{50}, used as a measure of the overall rate of differentiation (12), is 48 min at 28° (Fig. 5).

Extracts were prepared from cells at various times during differentiation, and the amount of tubulin antigen in each extract was determined. The amount of tubulin antigen present at 110 min, when cells have full-length flagella, was defined as 100%. Fig. 5 shows that antigen began to increase about 18 min after initiation of differentiation, and that the amount in whole cell extracts increased continuously throughout the time of measurement, which included the time when flagella were formed and during which the flagella grew to full length.

DISCUSSION

At least 70% of the outer doublet tubulin of *Naegleria* flagella is synthesized *de novo* during differentiation. This is a minimal estimate, since some or all of the remaining 30% of the radioactivity that was not chased is probably due to reutilization of amino acids released by protein breakdown. Previous immunochemical measurements showed that 97–98% of the tubulin antigen appears during differentiation (10). If appearance of tubulin antigen is due to synthesis of tubulin polypeptide, these two events should occur at the same time during differentiation. Tubulin synthesis begins at about 30 min in axenic cells that form flagella (T_{50}) in 80 min. Appearance of tubulin antigen begins at 18 min in bacteria-grown cells that form flagella at 48 min. In both cases the increase begins after 40% of the time required for differentiation has elapsed. The correlation of the beginning of synthesis and the first appearance of antigen suggests that these two events are the same, and that virtually all the flagellar tubulin is synthesized *de novo*.

Since the majority of flagellar tubulin is synthesized, the outer doublets cannot be assembled from a preexisting pool of tubulin subunits, such as might be expected if flagellar microtubules were assembled using mitotic tubulin. Thus, at least in this system, interconversion of tubulins does not account for the alternation of flagellum formation and mitosis. This result does not argue against other possible interconversions, such as between mitotic and cytoplasmic microtubules (16, 17).

Many biologists have emphasized the orderly and sequential events of "developmental programs," especially as seen in the appearance and disappearance of enzymes during development (18–20). The synthesis of tubulin during differentiation is clearly such a programmed event, perhaps the most rapid one requiring protein synthesis yet described in a eukaryote. Tubulin synthesis begins substantially before the assembly of flagella, or even of basal bodies (21). In contrast, in the formation of cilia on sea urchin embryos, Stephens (22) has found that tubulin synthesis takes place continuously throughout early development, although synthesis of other components of the embryonic cilia occurs at the beginning of cilium formation.

Once flagellar tubulin synthesis begins in *Naegleria*, it apparently continues even after tubulin molecules begin to be removed from the subunit pool by assembly of flagella (Figs. 3–5). More tubulin antigen is made than is assembled into flagella. If cycloheximide is added to cells at the time when half the cells have flagella, at a concentration that inhibits 98% of protein synthesis within 1 min, the cells continue differentiation and form full-length flagella (9). Yet at this point only about 40% of the tubulin antigen has been made (Fig. 5). In sea urchin embryos the tubulin pool is 3 to 4 times larger than is needed to make cilia on blastulae (22), and in both sea urchin embryos and *Chlamydomonas* flagellates regeneration of cilia or flagella in the near absence of protein synthesis indicates an excess of subunits (23, 24).

Differentiation in *Naegleria* is dependent on transcription (9). The increase in tubulin antigen does not occur if RNA synthesis is selectively inhibited with actinomycin D (25). This suggests a possible relationship between RNA synthesis and tubulin synthesis. A simple hypothesis is that transcription is needed to produce the messenger RNA for tubulin. Other explanations are possible, of course, including that interference with one step of the developmental program—RNA synthesis—prevents the occurrence of a subsequent step—tubulin synthesis. These possibilities focus our attention on one of the most challenging of many questions raised by the programmed synthesis of tubulin: what happens after the initiation of differentiation that leads, in as little as 18 min, to the synthesis of flagellar tubulin?

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