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Biological Factors Affecting Enflagellation of *Naegleria fowleri*

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Naegleria fowleri is a pathogenic amoeboflagellate that can be evoked to transform from amoebae to flagellates by subculture to nonnutrient buffer. More than half of the amoebae of strains KUL, nN68, and Lovell became enflagellated 300 min after subculture to amoeba-saline, whereas no amoebae of strains NF66, NF69, and HB4 did. *N. fowleri* nN68 enflagellated best when grown at 32 or 37°C and subcultured to amoeba-saline at 37 or 42°C. Amoebae from the stationary phase of growth enflagellated more readily than did actively growing amoebae. Incubation in expended culture medium from stationary-phase cultures enhanced the capability of growing amoebae to enflagellate after subculture to amoeba-saline. Enflagellation was more extensive when the population density in amoeba-saline did not exceed 2×10^5 amoebae per ml. Cycloheximide at 1 μg/ml and actinomycin D at 25 μg/ml inhibited growth of *N. fowleri* nN68. Cycloheximide at 0.5 μg/ml and actinomycin D at 25 μg/ml completely prevented enflagellation when added at time zero. Cycloheximide at 0.5 μg/ml, added 120 to 300 min after initiation of enflagellation, prevented further differentiation and caused existing flagellates to revert to amoeboid cells. Similarly, actinomycin D at 25 μg/ml, added 90 to 300 min after initiation of enflagellation, retarded differentiation and caused flagellates to revert. Radiolabeled precursors were incorporated into macromolecules during differentiation in nonnutrient buffer. Enflagellation of *N. fowleri* is a suitable model for studying regulation of a eucaryotic protist.

Members of the genus *Naegleria* are able to transform from feeding, dividing amoebae into nonfeeding, nondividing flagellates. The flagellate stage is transitory, and the flagellates revert readily to amoeboid cells (6). Enflagellation can be evoked reproducibly in the ubiquitous soil amoeba *Naegleria gruberi*. Critical factors for evoking enflagellation of *N. gruberi* include nutrient limitation, incubation temperature during differentiation, and growth conditions in axenic cultures (5). Factors not critical for evoking enflagellation of *N. gruberi* include stage of growth, growth temperature in association with bacteria in the range of 22 to 34°C, and population density during differentiation (4). These features, and the temporal reproducibility that has been attained, make *N. gruberi* a model for studies of gene expression and organellar synthesis occurring de novo during eucaryotic cell differentiation (6).

Precise experimental control of the enflagellation process of the pathogenic species *Naegleria fowleri* has not been reported heretofore, although the ultrastructure of enflagellation has been described (12). Amoebae of *N. fowleri* transform into elongated flagellates after subculture to nonnutrient buffer (13). The first alterations observed to date have been the loss of UV-absorbing material to the medium and a decrease in the number of vacuoles. Basal bodies, a rootlet, and flagella are formed de novo and relatively quickly after a period during which no other morphological changes obviously related to enflagellation are observed. Subsequently, the developing rootlet comes to lie in a furrow extending the length of the nucleus, which is located in the anterior end after the enflagellating cell becomes elongated (12). Morphological studies indicate that the transformation process, which occurs in the absence of exogenous nutrients, involves coordinated formation of new cell structures, presumably requiring new synthesis of protein, degradation of stored materials and macromolecules, and modification of cellular components such as cytoskeletal elements.

To study enflagellation of *N. fowleri* as a model for regulation of a eucaryotic protist, it is necessary to determine the factors which are critical for evoking the transformation from amoeboid to flagellated cells. The yield of flagellates must be high and reproducible. Means to

arrest or alter the course of enflagellation are needed to delineate stages of the process. Conditions for evoking enflagellation of some, but not other, strains of *N. fowleri* have been identified. Enflagellation is inhibited by actinomycin D or cycloheximide. Although exogenous nutrients are not required, selected precursors can be taken up and incorporated into macromolecules during enflagellation.

MATERIALS AND METHODS

The strains of *N. fowleri* used in this study were isolated from the spinal fluid of patients with primary amoebic meningoencephalitis (see Table 1). Stocks were grown axenically in Nelson medium (9): 2% (vol/vol) calf serum (GIBCO, Grand Island, N.Y.), 0.1% (wt/vol) glucose, 0.1% (wt/vol) liver digest (Panmede; Harrison and Crosfield, Bronxville, N.Y.), 120 mg of NaCl, 142 mg of Na_2HPO_4, 136 mg of KH_2PO_4, 4 mg of $MgSO_4 \cdot 7H_2O$, 4 mg of $CaCl_2 \cdot 2H_2O$ per liter of deionized water. Sterile medium was dispensed into tissue culture vessels (25 cm^2; Falcon Plastics, Oxnard, Calif.), inoculated to give about 2×10^4 amoebae per ml, and incubated at 37°C, unless otherwise noted, without agitation. Cell counts were made with an electronic cell counter (Coulter Counter model Z_{BI}; Coulter Electronics, Inc., Hialeah, Fla.) (15). Enflagellation was routinely evoked by removing the growth medium, rinsing the attached amoebae twice with amoeba-saline, and suspending the amoebae in amoeba-saline by vigorous agitation. Amoeba-saline contained 120 mg of NaCl, 142 mg of Na_2HPO_4, 136 mg of KH_2PO_4, 4 mg of $MgSO_4 \cdot 7H_2O$, and 4 mg of $CaCl_2 \cdot 2H_2O$ per liter of deionized water (11). In experiments requiring large numbers of amoebae, the attached cells were rinsed, harvested in a small volume of amoeba-saline, and diluted as appropriate into amoeba-saline previously warmed to 37°C. The culture vessels, containing 6 ml of cell suspension, were placed upright in a Gyrotory shaking water bath (model G76; New Brunswick Scientific Co., New Brunswick, N.J.) operated at 180 rpm. The time of the first rinse with amoeba-saline was defined as time zero. In experiments involving actinomycin D, the culture vessels were covered with aluminum foil to prevent photochemical degradation of the antibiotic.

The enflagellation process was monitored by light and phase-contrast microscopy of living specimens and of samples fixed with iodine solution (7). The number of amoeboid and elongated flagellated cells was ascertained in a total population of at least 100 cells.

Incorporation of radiolabeled precursors into enflagellating cells was measured after diluting 50-μl samples of enflagellating suspensions into 1 ml of 50 mM NaOH containing an excess of the corresponding unlabeled precursor and 100 μg of bovine serum albumin per ml. After incubation of the mixture at 37°C for 10 min, macromolecules were precipitated by addition of an equal volume of cold 25% trichloroacetic acid. After the precipitated macromolecules were chilled on ice for 1 h, precipitates were collected on glass-fiber filters (Whatman GF/A), rinsed with cold 5% trichloroacetic acid and with 95% ethanol, dried, and radioassayed by liquid scintillation spectrometry.

Chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise indicated. Radiolabeled compounds were obtained from New England Nuclear Corp. (Boston, Mass.). The specific activities of the radionuclides used were 320 Ci/mol for [U-^{14}C]leucine, 41 Ci/mol for [8-^{14}C]adenine and 1,095 Ci/mmol for [^{35}S]methionine. The ^{32}P was carrier free.

RESULTS

Twelve strains of *N. fowleri* were tested for ability to enflagellate after subculture to amoeba-saline (Table 1). No flagellates were detected in samples of three strains (NF66, NF69, and HB4), whereas three other strains (Lovell, KUL, and nN68) underwent extensive enflagellation. The three non-enflagellating strains (NF66, NF69, and HB4) were mixed in pairwise combinations with each other and with enflagellating strain nN68 to determine whether diffusible substances released by one strain stimulated or inhibited enflagellation by the other strain. No flagellates were observed in the pairwise combinations of the non-enflagellating strains, indicating that a diffusible factor, released by one strain, did not compensate for a factor limiting enflagellation by the other strain. The non-enflagellating strains were individually mixed with enflagellating strain nN68. Enflagellation in these pairwise mixtures was the same

TABLE 1. Strains of *N. fowleri* used, their sources, and their ability to enflagellate in nonnutrient buffer[a]

Strain	Source	% Enflagellation
Lovell	S. L. Chang (2)	65
KUL	E. van der Driessche (14), ATCC 30808	53
nN68	E. C. Nelson (3), formerly designated LEE, deposited as ATCC 30894	31
nN69-1	E. C. Nelson (3), formerly TY	22
nN69-2	E. C. Nelson (3), formerly WM	17
HB5	G. S. Visvesvara[b]	14
nN67	E. C. Nelson (3), formerly CJ	12
0359	J. B. Jadin (10)	2
GJ	C. Baro (16)	<1
HB4	R. B. Finley (18)	0
NF66	R. F. Carter (1)	0
NF69	M. Fowler (1)	0

[a] *N. fowleri* strains were grown in Nelson medium containing 2% calf serum at 37°C. The medium was removed, and the adherent cells were rinsed with amoeba-saline and covered with cold amoeba-saline to facilitate release of the amoebae. The suspended amoebae were diluted to 10^6 cells per ml and then shaken at 37°C. The % enflagellation given is the maximum value recorded for any hourly observation during the 8-h assay.

[b] Isolated by G. S. Visvesvara, Center for Disease Control, Atlanta, Georgia, in August 1977 from the spinal fluid of a patient in Edinburg, Texas.

as that for a comparable number of enflagellating nN68 cells alone, indicating that the non-enflagellating strains did not release a diffusible inhibitor and that strain nN68 did not release a diffusible factor needed by the non-enflagellating strains.

N. fowleri nN68 cells in actively growing cultures enflagellated only after a delay of 3 to 4 h after subculture to amoeba saline. Extensive enflagellation was achieved in a shorter period and with greater synchrony with amoebae in the stationary phase of growth (Fig. 1). The enhanced capability of stationary-phase cultures to enflagellate might reflect depletion of an inhibitor in the medium or production and release of a stimulating factor. The enflagellation ability of *N. fowleri* nN68 was altered by replacing the growth medium with fresh nutrient medium or with medium from stationary-phase cultures. Cultures of *N. fowleri* nN68 were grown to the mid-log phase (24 h). The nutrient medium of one set of cultures was replaced with medium from cultures in the early stationary phase of growth (74 h). Both sets of cultures were incubated for an additional 18 h at 37°C. After the second incubation period, the amoebae were subcultured to amoeba-saline at a population density of 2×10^5 cells per ml and shaken at 37°C. Maximum enflagellation achieved by the untreated logarithmic-phase culture (42 h) was 7%, whereas 42% of the amoebae from parallel cultures incubated in expended medium enflagellated. Expended culture medium enhanced the enflagellation ability of amoebae. Cultures of *N. fowleri* nN68 were also grown to the early stationary phase (74 h). The nutrient medium of one set of cultures was replaced with fresh medium. Both sets of cultures were incubated for an additional 18 h at 37°C. Maximum enflagellation achieved by the untreated stationary-phase culture (92 h) was 44%, whereas only 5% of the amoebae from parallel cultures incubated in fresh medium enflagellated. Fresh culture medium impaired the enflagellation ability of amoebae.

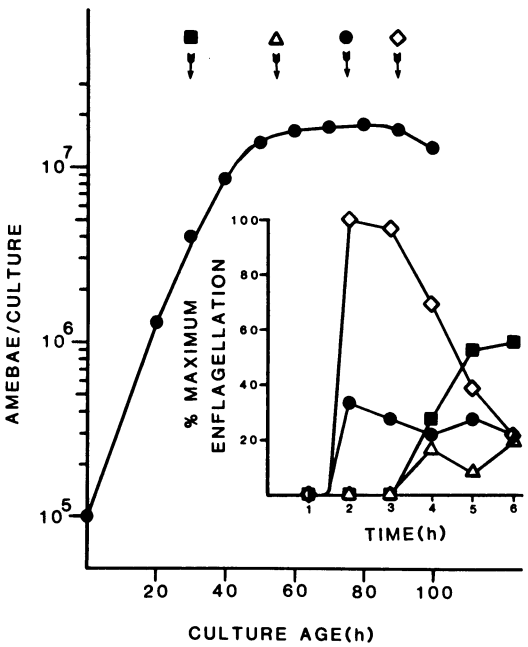

FIG. 1. Effect of growth phase before subculture to amoeba-saline at 37°C on enflagellation of axenic cultures of *N. fowleri* nN68 grown at 37°C. Amoebae at the indicated points (arrows) during axenic growth were harvested, washed, and suspended in amoeba-saline at equivalent cell densities, and enflagellation was monitored (inset) for 6 h.

The flagellate stage was transitory. Flagellates reverted to amoebae within minutes after agitation stopped. Even with agitation, flagellates reverted to amoebae. The proportion of flagellates present at a given time therefore was determined by the rate of flagellate formation and the rate of reversion to amoebae. Although cells from the stationary growth phase enflagellated more extensively than those from late-exponential-phase cultures, the former also reverted more rapidly (Fig. 1).

N. fowleri nN68 was grown in Nelson medium at 32, 37, or 42°C and then subcultured to amoeba-saline at 37°C. Amoebae grown at 32 and 37°C achieved maximum enflagellation (60%) 300 min after subculture, whereas amoebae grown at 42°C achieved only 18% enflagellation. *N. fowleri* nN68 grown at 37°C and then subcultured to amoebae-saline achieved maximum enflagellation (52%) after 360 min at 32°C, 60% enflagellation after 300 min at 37°C, and 65% enflagellation after 240 min at 42°C. Amoebae subcultured to amoeba-saline and incubated at 25°C did not form flagellates.

Growth of *N. fowleri* nN68 was retarded by 1 μg of cycloheximide per ml or 15 μg of actinomycin D per ml and markedly inhibited by 5 μg of cycloheximide per ml or 25 μg of actinomycin D per ml. Cycloheximide added at time zero completely prevented enflagellation at 0.5 μg/ml, and actinomycin D completely prevented enflagellation at 25 μg/ml. Cycloheximide added as late as 60 min after subculture to amoeba-saline completely suppressed the normal onset of enflagellation. Cycloheximide added 90 or 120 min after subculture to amoeba-saline did not retard enflagellation immediately; however, further enflagellation was inhibited 30 to 60 min after addition of the antibiotic. Cycloheximide did not retard reversion of existing flagellates to amoeboid cells. Cycloheximide added after 150 min not only prevented further enflagellation but

also accelerated the rate of reversion of the existing flagellates to amoeboid cells (Fig. 2). Actinomycin D added as late as 60 min after subculture to amoeba-saline completely suppressed the normal onset of enflagellation. Actinomycin D added after 90 min retarded enflagellation; however, the number of flagellates continued to increase for 30 to 60 min after addition of the antibiotic. Actinomycin D added after 180 min accelerated the rate of reversion of existing flagellates to amoeboid cells (Fig. 3). Light microscopic observation of enflagellating cultures confirmed that the flagellates reverted to amoeboid cells and that the antibiotics did not cause lysis of the differentiating cells.

The ability of enflagellating amoebae to incorporate selected radionuclides during incubation in amoeba-saline for 4 h at 37°C was assessed. Adenine, leucine, methionine, and P_i were incorporated into trichloroacetic acid-precipitable material (Table 2). The rate of incorporation was essentially linear with time, but the rates of incorporation of adenine and methionine by enflagellating suspensions (Table 2) were about 1/10 that of growing cultures (13). Most, but not all, of the $^{32}P_i$ label was soluble in hot 5% trichloroacetic acid. However, many phosphoproteins were synthesized by growing amoebae and by enflagellating *N. fowleri*. Little if any radioactivity was incorporated into trichloroacetic acid-precipitable material during a 4-h period when [^{14}C]glucose or [^{3}H]thymidine was supplied to the enflagellating cells. The number of *Naegleria* cells remained constant during the 4-h incubation in amoeba-saline; therefore, the failure to incorporate radiolabeled thymidine reflected the low level of DNA synthesis during enflagellation in nonnutrient medium.

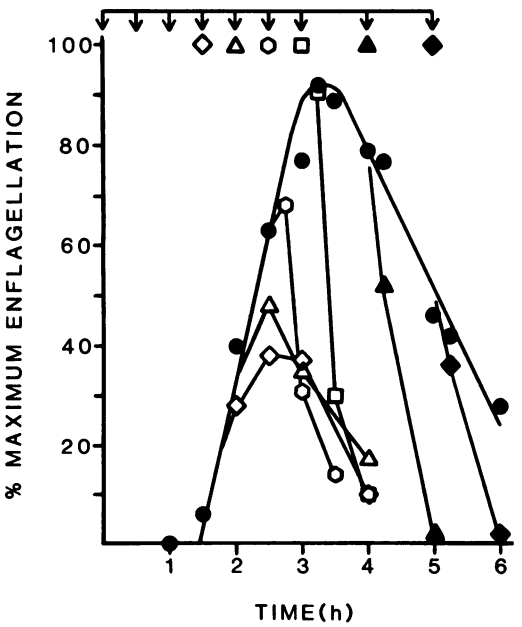

FIG. 2. Effect of delayed addition of cycloheximide (0.5 μg/ml) on enflagellation of *N. fowleri* nN68. Arrows denote time of addition of cycloheximide: after 1.5 h (◇), after 2 h (△), after 2.5 h (○), after 3 h (□), after 4 h (▲), and after 5 h (◆). No flagellates were observed in flasks receiving antibiotic at time zero, after 0.5 h, and after 1 h. Values are percentages of the maximum enflagellation in control flasks (50%).

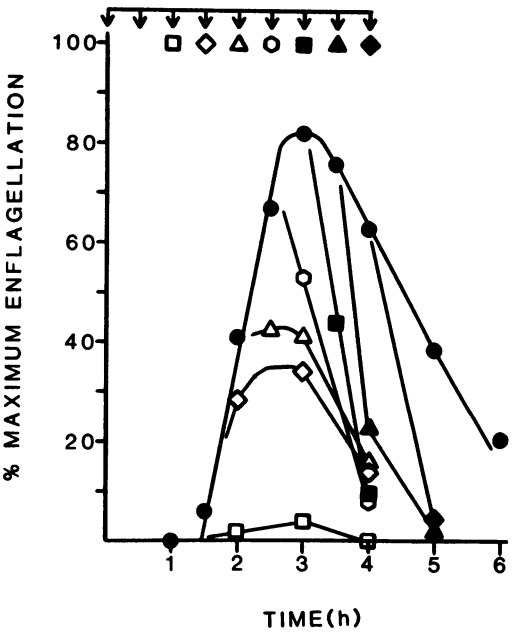

FIG. 3. Effect of delayed addition of actinomycin D (25 μg/ml) on enflagellation of *N. fowleri* nN68. Arrows denote time of addition of actinomycin D: after 1 h (□), after 1.5 h (◇), after 2 h (△), after 2.5 h (○), after 3 h (■), after 3.5 h (▲), and after 4 h (◆). No flagellates were observed in flasks receiving antibiotic at time zero and after 0.5 h. Values are percentage of the maximum enflagellation in control flasks (49%).

Several variables that might affect enflagellation were examined. Neither the number of rinses with amoeba-saline nor the volume of rinses altered the yield of flagellates or the rate of flagellate formation. Delays between rinsing and suspension in amoeba-saline adversely affected yield and rate of enflagellation. The amoebae could be concentrated by sedimenting them by centrifugation or by releasing attached amoebae into a small volume of amoeba-saline by chilling the culture at 5°C for 10 min. The amoebae could be pipetted vigorously and dispersed by vigorous agitation with a Vortex mixer without adversely affecting their ability to enflagellate. The extent of enflagellation of strain nN68 was suppressed at population densi-

TABLE 2. Incorporation of selected radiolabeled precursors into macromolecules of enflagellating *N. fowleri*[a]

Precursor	Input (μCi/ml)	Incorporation	
		Counts[b]	%
[8-^{14}C]adenine	6.7	4.0×10^4	0.6
[*U*-^{14}C]leucine	7.4	3.6×10^4	0.5
[^{35}S]methionine	54	5.7×10^4	0.1
$^{32}P_i$	113	1.7×10^5	0.2

[a] *N. fowleri* amoebae from early stationary-phase cultures were harvested, rinsed, suspended in amoeba-saline at a population density of 5×10^5 cells per ml, and shaken at 37°C for 4 h. The indicated radionuclide was added at time zero. Incorporation of radiolabel was assayed at time zero and after 4 h (13).

[b] Count min^{-1} per 10^6 cells, corrected for background.

ties greater than 5×10^5 cells per ml of amoeba-saline. Amoebae at population densities of 2×10^4 to 2×10^5 cells per ml of amoeba-saline enflagellated to the same extent (Fig. 4).

DISCUSSION

N. gruberi (6) and *N. fowleri* (12) proceed through similar stages leading to flagellate formation: (i) initiation by nutritional step down, (ii) appearance of basal bodies, (iii) formation of flagella and rootlets, and (iv) cell elongation. Although there are many similarities in enflagellation of the two species, there are a number of interesting differences in the regulation and the program of the two processes, such as the differences in their temporal program for enflagellation. Both *N. gruberi* flagellates (4) and *N. fowleri* flagellates can revert to amoebae almost instantly. This rapid reversion to motile amoebae indicates that the amoeboid motility system has persisted in some latent form in the flagellated cells.

Growth temperature is an important variable for differentiation by axenically grown cultures of *N. gruberi* (5) and of *N. fowleri*. *N. gruberi* enflagellates extensively when subcultured to the same or a lower temperature (7), whereas *N. fowleri* nN68 enflagellated with higher yields and greater synchrony when subcultured to the same or a higher temperature. An important determinative factor in *N. fowleri* enflagellation appeared to be population density during growth and differentiation. Population density during differentiation and growth stage do not appear to be critical variables for *N. gruberi* enflagellation (6), although some correlations have been observed between the pH of late-logarithmic-phase axenic cultures of *N. gruberi* and the extent of enflagellation (5). The experimental cues for enflagellation are suspension, dilution, and agitation of amoebae in nonnutrient amoeba-saline.

Several lines of evidence affirm that *N. fowleri* nN68 was synthesizing many macromolecules during the enflagellation process: the antibiotics cycloheximide and actinomycin D inhibited enflagellation, and radiolabeled precursors were incorporated into macromolecules. A wide spectrum of proteins is being synthesized during enflagellation (17), indicating that the enflagellation process does not involve a general shutdown of protein synthesis. Moreover, no limited number of radiolabeled polypeptides dominates the complement of newly synthesized macromolecules (17), indicating that preferential new syntheses of one or a few proteins is not occurring during enflagellation.

Both *N. gruberi* (8) and *N. fowleri* synthesize protein and RNA during differentiation. For *N. gruberi* however, cycloheximide is no longer able to block differentiation if added after a specific time (ca. 60 min). In contrast, cycloheximide not only stopped further differentiation of *N. fowleri*, but also accelerated the rate of reversion of existing flagellates to amoebae, even when the antibiotic was added at advanced stages of the enflagellation process. Perhaps continued protein synthesis is needed to maintain the flagellate morphology. For both *N. gruberi* and *N. fowleri*, growth-inhibiting concentrations of actinomycin D, added at an appropriate time after subculture to conditions that

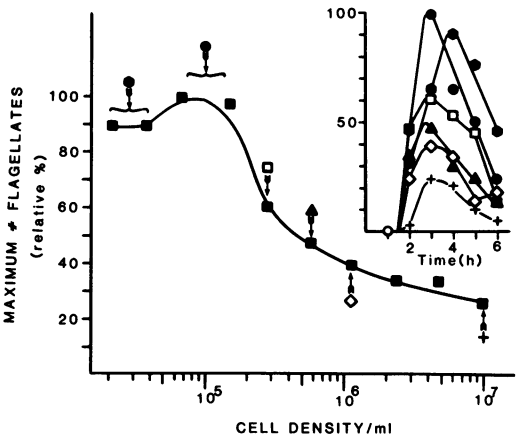

FIG. 4. Effect of population density during differentiation in amoeba-saline at 37°C on enflagellation ability of *N. fowleri* nN68 grown axenically at 37°C. Amoebae from stationary-phase cultures were harvested, washed, and suspended at the cell density indicated (arrows). Maximum enflagellation observed during 6 h is expressed relative to that of cells at 7×10^4 amoebae per ml (40% enflagellation). Inset: Time course of enflagellation for cell suspensions at the indicated densities: 2×10^4 or 3.5×10^4/ml (●), 7×10^4 or 1.4×10^5/ml (●), 3×10^5/ml (□), 6×10^5/ml (▲), 1×10^6/ml (◇), and 1×10^7/ml (+).

elicit enflagellation, allow limited but continued enflagellation. However, actinomycin D arrested enflagellation of *N. fowleri* at all points during enflagellation, indicating that mRNA is not present in sufficient concentration to complete enflagellation of all cells, that mRNA is degraded before all necessary protein synthesis is completed, or that secondary effects of drug inhibition limit the biosynthetic capabilities of the differentiating cells.

ACKNOWLEDGMENT

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