Leucine Incorporation into the Membranellar Bands of Regenerating and Nonregenerating Stentor

Abstract. *Autoradiographs of membranellar bands isolated from regenerating and nonregenerating stentors labeled with tritiated leucine indicate that the synthesis of proteins of the oral apparatus is continuous in* Stentor *and is not initiated at the time of regeneration.*

The oral apparatus of the large ciliate *Stentor coeruleus* consists of a band of membranelles ending in an oral pouch and gullet. The membranelles are composed of brushes of cilia attached at their bases to corresponding rows of kinetosomes which are linked together by fibrous connections (*1*). On the lateral surface longitudinal rows of cilia (kineties), alternating with stripes of blue-green pigment granules, extend from the anterior feeding organelles to the posterior holdfast. The pigment stripes are graded in width around the organism; the site at which broad and narrow stripes intersect is called the "locus of stripe contrast." When oral structures are removed or irreparably damaged, a new oral apparatus begins to form at the locus of stripe contrast; large numbers of kinetosomes appear, sprout cilia, and become aligned in rows to form a new set of oral membranelles. Later, a gullet forms at the posterior part of the primordium as it migrates to its final anterior position. The entire process lasts approximately 8 hours at 22°C.

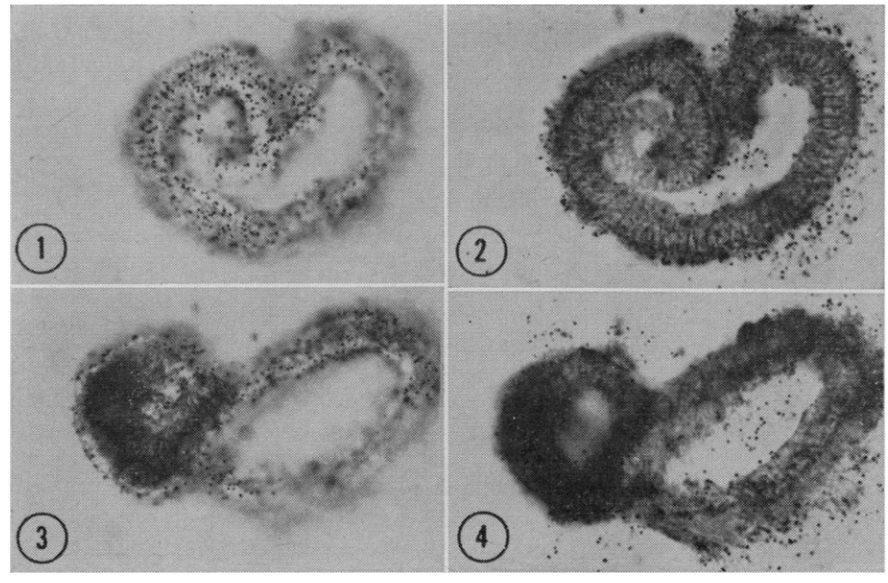
The cilia of *Tetrahymena* are composed mainly of protein (*2*); it is, therefore, not surprising that the oral apparatus and the oral primordium (when present) are the most prominent and deeply stained cytoplasmic structures of stentors stained for protein with mercuric bromphenol blue (*3*). The somatic kinetosomes, kinetodesmata and cilia of *Stentor* also stain very deeply with bromphenol blue.

In order to determine whether the proteins which constitute the major part of the developing organelles are synthesized *de novo* during regeneration and, if so, whether nonregenerating organisms can also synthesize these proteins, regenerating and nonregenerating stentors were exposed to tritiated leucine; their membranellar bands were isolated, and the incorporation of the label was determined by autoradiography.

Stentor coeruleus was grown as described (*3*) except that a modified Peters' solution (*4*) adjusted to *p*H 7.5 with 1N HCl served as inorganic medium. The organisms were left overnight in Peters' solution to deplete their food reserves and prevent initiation of division during the experiment. They were then incubated for 1 hour in Peters' medium containing tritiated leucine (50 μc/ml) (*5*); the isotope was not supplied during regeneration because labeled compounds are taken up poorly by differentiating stentors (*6*). The stentors were rinsed by centrifugation in ten changes of inorganic medium and divided into two groups. Group I was given no further treatment; Group II was transferred briefly to 2 percent urea, which induces oral regeneration by causing stentors to shed their membranellar bands (*7*). The shed bands consist of membranelles attached to a continuous basal ribbon which holds them together and which must, therefore, contain the kinetosomes and their fibrous interconnections. Approximately 8 hours later, after the organisms of group II had completed regeneration, the organisms of both groups were briefly treated with 2 percent urea containing 0.1 percent lithium chloride (*8*) and their membranellar bands were collected. Since the membranelles begin to disintegrate soon after isolation, immediately after the bands were shed they were transferred into cold calcium-formol fixative (*9*) and fixed for a minimum of 24 hours. They were then transferred through three changes of distilled water, dried down on gelatin-coated microscope slides and extracted for 15 minutes at 90°C in 5 percent TCA (trichloroacetic acid) to remove unincorporated label. The slides were rinsed in distilled water, dried, covered with Kodak NTB2 autoradiographic emulsion and exposed for 6 weeks at room temperature.

The autoradiographs revealed labeling of membranellar cilia and basal ribbons in both groups (Figs. 1 to 4). The bands of newly regenerated organisms generally appeared to be more strongly labeled than those isolated from stentors which had not replaced their oral structures. Large variations in the degree of labeling were found in both groups, probably because of individual differences in the uptake of the isotope. Three separate experiments using stentors from three different cultures yielded the same results.

The data could be subject to errors caused by cytoplasmic contamination



Figs. 1–4. Autoradiographs of isolated membranellar bands. Fig. 1. Label over basal ribbon of newly regenerated band. Fig. 2. Label over membranellar cilia of newly regenerated band. Fig. 3. Label over basal ribbon of nonregenerated band. Fig. 4. Label over membranellar cilia of nonregenerated band.

Table 1. Number of oral membranelles in ten newly divided and ten 24-hour-old stentors. Duplicate counts were made on each organism.

Newly divided	24-hour-old
204	192
205	183
182	230
191	230
197	227
211	219
236	217
232	214
207	218
213	216
257	195
256	196
202	211
203	211
233	233
234	232
209	260
207	263
202	203
203	198

or by nonspecific adsorption of isotope. However, there was no microscopic evidence of contaminating particles. The membranellar cilia could not have become labeled through contamination since they are extracellular structures, shed without lysis of the organisms. Extraction with hot 5 percent TCA should have removed all unincorporated label, but the possibility that labeling might be caused by nonspecific adsorption of isotope was examined. Isolated membranellar bands were fixed in formalin, rinsed thoroughly in distilled water, incubated for 4 hours in tritiated leucine (100 μc/ml), and extracted with hot 5 percent TCA. When examined by autoradiography, these bands showed no signs of radioactivity.

Indirect evidence suggests that oral regeneration in *Stentor* requires the synthesis of RNA. Enucleation (*10*) or treatment at early stages of regeneration with inhibitors of RNA synthesis (*11, 12*) blocks regeneration; however, these treatments are ineffective after the oral membranelles are fully formed and only the gullet and oral pouch remain to be invaginated (*10, 12*). This latter finding suggests that at least some of the RNA required for regeneration may be messenger RNA coding for oral apparatus proteins. That membranellar bands formed in the presence of tritiated leucine contain the isotope suggests that oral apparatus proteins are indeed synthesized *de novo* during regeneration.

The data provide evidence on the question of whether the appearance of new oral structures during regeneration results from the activation of genes coding for oral apparatus proteins. The incorporation of tritiated leucine into membranellar cilia and basal ribbons of nonregenerating stentors suggests that, while protein synthesis may be one prerequisite for oral regeneration, the appearance of new kinetosomes and membranellar cilia at this time does not result from a newly acquired capacity for synthesis of the constituent proteins.

Incorporation of leucine into the oral structures of nonregenerating stentors may represent turnover of proteins in pre-existing oral structures, addition of new cilia and kinetosomes to pre-existing membranelles, or formation of new membranelles. In order to investigate the last of these possibilities, small (newly divided) and large (24-hour-old) stentors were fixed by the method of Repak and Hirshfield (*13*), and the number of membranelles determined either by counting them directly or by counting the rows of pigment granules which alternate with the membranelles. Since it proved impossible to count membranelles or pigment bands in the deeper spirals of the gullet, the results presented in Table 1 are not strictly accurate. However, they are accurate enough to show that the number of membranelles does not double during the cell cycle and to suggest strongly that the feeding structures enlarge during growth by changes in the spacing of the original membranelles. The possibility remains that new cilia, kinetosomes and fibres are added to the original membranelles as the organism grows. However, Tartar has observed the membranelles of large and small stentors to be approximately equal in width and length (*10*). It therefore appears more likely that labeling of the membranellar band during growth is caused by turnover of protein within pre-existing organelles.

Results similar to the present findings have recently been obtained with *Tetrahymena*. Williams and Zeuthen (*14*), using heat-synchronized *organisms*, showed by autoradiography that the kinetosomes and fibers of the oral apparatus incorporate tritiated leucine in the absence of oral differentiation. Recent work by Child (*15*) suggests that *Tetrahymena* can also synthesize ciliary proteins in the absence of oral differentiation. On the other hand the flagella of *Astasia* and *Ochromonas* incorporate tritiated leucine only during flagellar regeneration (*16*). This difference can perhaps be explained by the fact that ciliates are constantly adding new cilia and kinetosomes to the somatic kineties during interphase (*17*). Newly synthesized ciliary and kinetosomal proteins may be constantly available for exchange with similar molecular species in preformed oral organelles. Cilia are known to be dynamic structures which can be resorbed (*10*) and which are capable of regeneration if removed or transected (*18*).

Although my data indicate that stentors synthesize ciliary proteins continuously, oral regeneration presumably requires the production of more oral apparatus proteins than are utilized during normal growth. However, stentors can produce extra sets of feeding organelles with no retardation if extra loci of stripe contrast are present (*10*); this finding suggests that the principal factor in determining the appearance of oral structures is not the quantity of protein synthesized, but the creation of a demand for protein at specific sites.

My results provide additional evidence that induced synthesis of oral apparatus proteins does not occur during oral differentiation in ciliates. The important regulatory event must involve other conditions which promote extensive multiplication of kinetosomes at the locus of stripe contrast and the outgrowth of fibers and cilia from them. In *Stentor*, pre-existing oral structures normally inhibit production of kinetosomes and cilia at the locus of stripe contrast (*19*); Tartar's finding that regeneration will occur if the oral apparatus is reversed *in situ* (*10*) suggests that inhibition does not occur because the two sites are competing for precursors.

NOËL DE TERRA
Laboratory of Nuclear Medicine and Radiation Biology, Department of Biophysics and Nuclear Medicine, School of Medicine, University of California, Los Angeles

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One-Way Stimulation in Mixed Leukocyte Cultures

We have developed an improved method for the mixed leukocyte culture test. Control values, as determined by rates of incorporation of thymidine, are very low, allowing evaluation of low levels of stimulation in homologous cell mixtures. One-way stimulation is assayed by treating the cells of one individual with mitomycin C; treated cells cannot respond (incorporate thymidine) but can still stimulate homologous untreated cells to do so.

Determination of histocompatibility in man by use in vitro of mixed leukocyte cultures (MLC) is receiving attention. The original observation was that mixtures of leukocytes from two genetically unrelated individuals resulted in mutual stimulation, evidenced by enlargement and mitosis of lymphocytes in culture. The method seemed to have potential as a measure of histocompatibility because (i) mixtures of cells from identical twins did not result in stimulation (*1, 2*), (ii) mixtures of cells from related individuals resulted in less stimulation, on the average, then mixtures of cells from unrelated individuals (*3*), and (iii) the histocompatibility status of one set of three individuals, based on MLC determinations, correlated with their compatibilities determined by the third-man test (*2*). Other reported data (*4, 5*) suggest that the test does provide some measure of histocompatibility in man.

Some undesirable features in the method described by Bach and Hirschhorn (*2*) have become apparent: High control values in cultures containing cells from one individual make it difficult to evaluate the significance of low levels of stimulation in mixed cultures; although the values are reduced by use of human serums instead of fetal calf serum (*6*) in the medium, control values still represent up to 20 percent of values obtained in mixed cultures of incompatible cells. Furthermore, cells from both individuals under test are able to respond to each other; it is obviously desirable to measure one-way stimulation (*7*)—wherein the contribution of each individual's cells to the total response can be measured independently.

We now describe an improved method for MLC testing in which (i) control values, as determined by rates of thymidine incorporation, are very low —in many instances zero; and (ii) one-way stimulation is assayed by treating the cells of one of the test subjects with mitomycin C to prevent DNA replication (*8*). Such treatment prevents the cells from acting as "responding cells" (responding to homologous tissue by enlarging and incorporating thymidine) without preventing them from acting as "stimulating cells" (stimulating homologous cells to respond) (*9*).

Freshly drawn heparinized blood is allowed to sediment in 25- by 150-mm glass tubes for 2 hours at 37°C; subsequent procedures preceding culture are at room temperature. The plasma is centrifuged for 10 minutes at 150*g* to obtain leukocytes; the supernatant is recentrifuged for 10 minutes at 1000*g* to provide cell-free plasma. Cells to be tested as "responding cells" are suspended in Eagle minimal essential medium (*10*) modified for suspension culture (MEM-S, Grand Island Biological) and supplemented to contain glutamine, 2 m*M*; penicillin, 100 units per milliliter; streptomycin, 100 μg/ml; and 20 percent autologous cell-free plasma.

Cells to be used as stimulating cells are suspended at a concentration of 2 to 10 $\times$ 10^6 leukocytes per milliliter in MEM-S containing 10 percent autologous plasma, incubated for 20 minutes at 37°C with mitomycin C at 25 μg/ml, twice washed in Hanks buffered salt solution containing 10 percent plasma from the donor of the responding cells, and then suspended in MEM-S containing 20 percent plasma from the donor of the responding cells. Stimulating cells are mixed with responding cells to give final cell concentrations of, respectively, 7 to 10 $\times$ 10^5 (leukocytes) and 5 $\times$ 10^5 (lymphocytes) per milliliter. The mixture is immediately distributed in 2.5-ml volumes in 16- by 100-mm glass tubes with metal closures, and incubated at 37°C in a humidified 4 percent CO_2 atmosphere for 7 days.

After approximately 160 hours of incubation, 2 μc of tritiated thymidine (specific activity, 1.9 c/mmole; Schwarz BioResearch) is added to each culture. Replicate cultures are harvested 1.5, 3.5, and 5.5 hours later by addition of 1000-fold excess of nonradioactive thymidine in 0.1 ml of normal saline, immersion of the culture tubes in ice, centrifugation for 10 minutes at 1000*g*, and freezing of the cell sediment at −20°C. To determine incorporation of thymidine the cultures are thawed, 5 ml of cold 5-percent trichloroacetic acid is added to each, and the tube contents are mixed on a Vortex mixer. Ten minutes later the precipitate is recovered by centrifugation, dissolved in 1 ml of 0.1*N* NaOH, and precipitated again with 4.5 ml of 6.7-percent trichloroacetic acid. The acid-precipitation procedure is repeated twice again. The final precipitate is dissolved in 0.1 ml of tetraethylammonium hydroxide, mixed with scintillation fluid (*11*), and counted; the results are expressed as counts per minute (cpm) in each sample. Reproducibility of the assay was determined with 150 mixtures, each mixture being cultured in triplicate; 82 percent of all triplicates showed counts within $\pm$ 12 percent of their mean; 97 percent, within $\pm$ 18 percent. Six of the 450 samples were disregarded because they deviated from the mean of the triplicates by more than 60 percent (always on the low side); they probably reflected some unusual technical accident.

Responding cells (A), incubated with autologous mitomycin C–treated stimulating cells (A_m), serve as a control for homologous mixed cultures (for example, AB_m, AC_m, and such); thus is enabled comparison of the reactions of cells of one individual (a potential recipient) with reactions of the homologous cells of a series of other individuals (potential donors).

Fig. 1*A* shows the results of culturing together the responding cells (F) of one individual with his own stimulating cells (F_m) and with those from another individual (G_m). Label was added after 160 hours and triplicate cultures were harvested at the times shown. Four slopes representing rates of incorporation were obtained. While the rate in