in a final volume of 100 μl; (iv) for α-galactosidase: colorimetrically, 6 μmole of *p*-nitrophenyl-α-D-galactoside, 20 μmole of acetate buffer (*p*H 4.5), and leukocyte enzyme extract in a final volume of 500 μl; and fluorometrically, 1 μmole of methylumbelliferyl-α-D-galactoside, 15 μmole of acetate buffer (*p*H 5.0), and leukocyte extract in a final volume of 200 μl. All assays were done at 37°C. At the end of the incubation time (2 hours for α-galactosidase, 30 minutes for the other three enzymes), the mixtures were diluted to 3 ml with 0.1*M* diaminoethane buffer (*p*H 10.5). The extinction of the *p*-nitrophenol at 410 nm and the fluorescence of the methylumbelliferone (excitation, 360 nm; fluorescence 450 nm) could then be measured. The activity of the four enzymes from leukocytes of 15 normal males and of patients (from two families) with Fabry's disease is shown in Table 1.

The α-galactosidase activity was absent from the male patients' white cells, whereas the activity of the other three lysosomal enzymes was normal or higher than normal. Mixing a leukocyte homogenate of a control with that of a patient showed that an inhibitor was not responsible for the diminished activity in the patients' white cells. These findings support the assumption that the accumulated glycolipid in Fabry's disease has a terminal α-galactose residue. In fact, the exact conformation—α or β—of the glycosidic bonds in the accumulated ceramide trihexoside is difficult to establish and, to our knowledge, has not been determined.

Because the disease is transmitted by a sex-linked recessive gene, the α-galactosidase activity was measured in the leukocytes of other members of both families. Four females, presumed to be carriers, showed reduced enzyme activities (Table 1). This assay should therefore provide a simple and reliable method for the diagnosis of Fabry's disease in the clinical and in the carrier state.

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Transformation of Tetramitus Amebae into Flagellates

Abstract. *Methods have been developed for controlling the transformation of* Tetramitus rostratus, *a protozoan able to reproduce either as an ameba or as a flagellate. An essential condition for transformation is a low concentration of oxygen in the environment.*

The amebo-flagellate *Tetramitus rostratus* can reproduce stably in either of two distinct phenotypes. For 70 years after the organism was discovered in 1852 (*1*), it was known only as a flagellate. Flagellates of *T. rostratus* have four anteriorly directed flagella, and a characteristic shape which includes a cytostomal groove and a beaklike rostrum. The flagellates, which reproduce by binary fission, have been unhesitatingly classified among the Mastigophora. Then in 1922 Bunting (*2*) found an ameba, whose appearance and movement was similar to many small (10 to 20 μm) amebae, which reproduced by binary fission, and which would be placed in the class Sarcodina. Bunting's amebas, however, could transform into flagellates that were indistinguishable from *T. rostratus*. Although the ability of this organism to alternate between two very different but stable phenotypes offers numerous opportunities for study, *T. rostratus* remains little known. One reason for this is that it has been impossible to control the phenotypic changes.

There have been four previous studies of conditions influencing the ameba-to-flagellate transformation. Three used standing cultures in complex media, with bacteria present (*2–4*). In the most recent of these, Outka (*4*) found that a threshold population density of amebae was necessary for transformation. He observed no flagellates until 24 hours after inoculation, but eventually obtained up to 80 percent flagellates. Since flagellates can reproduce, it is difficult in such long-term experiments to determine what proportion of the flagellates result from transformation and what proportion from differential growth of transformed cells. Brent (*5*) made the only study of transformation in nonnutrient buffer. He observed flagellates within 2 hours, to a maximum of 3 percent by 7 hours. Brent found more flagellates at higher population densities, which indicated as did Outka's experiments that an interaction of amebae is important for transformation. Brent sought, but did not find, a "flagellating substance."

In our initial attempts to control *Tetramitus* transformation, we could obtain only about 1 percent flagellates in buffer, or up to 50 percent in nutrient media, where some of the flagellates undoubtedly came from growth (*6*). We confirmed that higher population densities of amebae favor transformation. Although at suitable population densities, standing, undisturbed cultures would transform, agitated cultures would not. The requirement for a stagnant environment was unfortunate, since transformation could best be studied with cells in homogeneous suspension, so that the environment around each cell would be uniform and so that random samples could be removed to measure the phenotypic change (compare *7*). Efforts were made to find conditions for transformation of agitated cultures. Agitation prevented transformation only when it resulted in continuous exchange of gases between culture and environment. Sealed liquid cultures—for example, in syringes—transformed if the population density was sufficient regardless of whether or not they were agitated. This suggested that the flagellating substance was volatile. All attempts to collect the substance from one transforming culture and introduce it into another, using Loomis' "air bridge" method (*8*), were unsuccessful. The failure to find a volatile substance led to the hypothesis that the flagellating substance might be something taken from the environment rather than something added to it. Oxygen seemed a likely candidate. Oxygen would be expected to disappear from standing cultures, or sealed agitated cultures, at a rate proportional to the population density of amebae. When this hypothesis was tested, using agitated suspensions of amebae in low-oxygen atmospheres, most cells transformed fairly rapidly.

The best conditions available at present give transformation comparable to

the example shown in Fig. 1. Amebae of *T. rostratus* strain TB-2, a clone derived from a culture supplied by Brent, were grown at 28°C on Brent's agar medium in association with *Escherichia coli* strain CS101. The agar medium, containing 0.5 percent Bacto-peptone (Difco), 0.5 percent yeast extract (Difco), and 1.5 percent Bacto-agar, was poured into 10-cm petri dishes. About 10^5 amebae and 10^8 bacteria were spread over the surface of the agar, and the culture was incubated overnight at 28°C. The amebae were harvested while they were still growing exponentially, when they had just begun to clear the bacterial lawn. At this time there were about 5×10^6 amebae per dish. The amebae and bacteria were suspended in 10 ml of Brent's buffer (6.7 m*M* potassium phosphate in demineralized water, *p*H 7.3), sedimented for 90 seconds at 650*g*, and the supernatant, which contained most of the bacteria, was discarded. The amebae were resuspended, and washed by two more centrifugations. After the final centrifugation the amebae were resuspended in Brent's buffer, adjusted to a concentration of 1.7×10^5 per milliliter, and 8 ml was placed in a 125-ml Erlenmeyer flask. The flask was capped with a sleeved rubber stopper (A. H. Thomas No. 8826, 16 mm), and the gaseous environment of the flask adjusted (*9*). The flask was evacuated and flushed with nitrogen twice, and after a third evacuation the flask was filled with a mixture of 0.4 percent O_2 and 0.4 percent CO_2 in N_2, at atmospheric pressure. The flask was then placed in a 30°C water bath and shaken reciprocally at 125 one-inch oscillations per minute. It took 13.5 minutes from suspension to water bath; the time for transformation is measured from the time the flask was placed in the bath. Samples were removed from the culture with a 19-gauge needle and fixed in Lugol's iodine (*9*). Transformation was measured by counting the percentage of cells with flagella.

This protocol, followed closely, has given reproducible results. No flagellates have ever been observed until 2.5 hours after lowering the oxygen concentration (compare *5*). The proportion of cells with flagella increases during the ensuing 2 hours to near maximum by 4.5 hours. It has not been possible regularly to seduce more than 80 percent of the cells to transform.

Low concentration of oxygen seems to be the crucial factor, the flagellating substance of Brent. Transformation in a low-oxygen environment is relatively independent of population density and of agitation. No transformation has ever occurred in agitated cultures exposed to air (about 21 percent O_2) or in the total absence of oxygen, regardless of the population density. An atmosphere of about 0.3 to 0.4 percent O_2 in N_2 seems to be optimum. Other factors influence the competence of amebae to transform. These include growth medium, bacterial associate, and the growth phase of the amebae. The addition of CO_2, though not required, increases the proportion of transforming cells. If amebae are stored in nonnutrient buffer for several hours before the oxygen concentration is lowered, few transform, which may explain why Brent obtained only 3 percent flagellates.

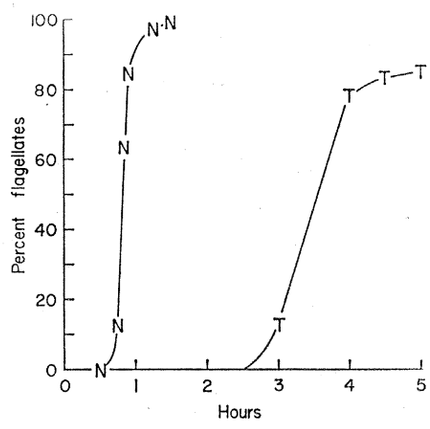

Fig. 1. Transformation of *Naegleria* and *Tetramitus* in agitated suspensions in nonnutrient buffer at 30°C, under conditions favorable for each organism but otherwise comparable. *T, T. rostratus* TB-2 was transformed with a gaseous environment of 0.4 percent O_2 and 0.4 percent CO_2 in N_2, as described in the text. *N, N. gruberi* NEG (*9*) was transformed with the flask open to room air, under conditions described previously (*7*).

The role of an oxygen-deficient environment in transformation is unknown, but may in some way be related to the ecology of the organism. *Tetramitus* flagellates have usually been found in stagnant aquatic environments and stagnant laboratory cultures (*9*). Hollande (*3*, p. 177) found that strong aeration resulted in the disappearance of flagellates, but concluded that "the impoverishment of the culture medium of oxygen [by sealing the culture] is not, in itself, a sufficient factor to provoke the transformation of the ameba into a flagellate." Since many factors interact together, and low oxygen is a short step—in a sealed culture—from anaerobiosis, it is not surprising that Hollande did not obtain transformation of sealed cultures.

Transformation of *T. rostratus* is less rapid than that of the related amebo-flagellate *Naegleria gruberi*, even under similar conditions of temperature and agitation (Fig. 1). This difference has persisted, even though transformation of both organisms is measured as the development of flagella, and even though the amebae of both grow at about the same rate (minimum doubling time about 2 hours). *Naegleria* transformation depends, operationally, on suspension of the amebae in nonnutrient buffer. It does not depend on a low oxygen concentration. *Naegleria* transformation is easier to control, and can be made quite synchronous (*7*, *9*). But whereas *Tetramitus* flagellates form a mouth and can reproduce as flagellates, *Naegleria* forms flagellates which swim for a few minutes or days, depending on conditions, but do not feed or reproduce and invariably revert to amebae.

Considerably more is involved in the differentiation of definitive *T. rostratus* flagellates than simply the development of flagella, and other changes accompany and follow flagellum formation under the conditions described. Transforming amebae round up before flagella are seen. The spherical cells usually form only two flagella; only later do cells have four flagella. As the flagellates acquire the characteristic shape and organelles of *T. rostratus*, a division occurs. Possibly this division is meiotic, and perhaps it can be followed by syngamy. Controlled transformation provides the tool for studies to elucidate the full life cycle, to obtain reproducing flagellates, to control the reversion of flagellates to amebae, and to learn what is involved in the alternation of stable phenotypes in this organism.

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