Protoporphyrin Production of *Tetrahymena geleii*. (20404)

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1. *Growth of the organism and formation of the pigment.* Cultures of *Tetrahymena geleii,* strain W,† which had been grown for many years in daylight as a food for *Tokophrya infusionum*(1,2) were observed about a year ago to produce a red pigment when grown in the dark. The cultures were grown in test tubes at 25°C on an agar medium containing 2% proteose peptone, 1.5% agar, 0.25% dextrose, 0.025% $(NH_4)_2HPO_4$ and 0.0005% thiamin. The pigment in Tetrahymena was observed, by microscopical examination, to be enclosed in small vacuoles about 3-4 μ in diameter. The greatest accumulation of vacuoles with red pigment was located in the posterior part of the body. It was observed also that some of the pigment, still enclosed in a vacuole, was excreted from the body through the cytopyge. Once outside the body of the Tetrahymena the pigment collected at the bottom of the water of condensation in the test tubes and also along the surface of the slant where the agar, water of condensation, and the inner side of the tube meet. Individuals containing the red pigment were found to be of greater size than those without it.

Growth on a solid medium appears to be necessary for the production of the pigment. In liquid medium the red pigment was absent from the organism even when a culture with organisms containing the red pigment was used as inoculum for subcultures in a liquid medium. However, the occurrence of individuals with red pigment was greater in subcultures made from cultures grown in a liquid medium than in those transferred from a solid medium to another solid medium. The number of organisms containing red pigment was greatest in a culture 5-8 days old. As the cultures became older the color turned somewhat brownish. The red pigment when excited by U.V. light of 400 mμ fluoresced red, a property of porphyrin compounds. The younger and older cultures appeared to contain only one major pigment, identified subsequently as protoporphyrin.

Fluorescing porphyrin pigments are known to be photodynamically active(3) and in the presence of visible light might be expected to be inhibitory to growth. Very dilute protoporphyrin solutions have been reported by Fischer and Schneller(4) to act as photosensitizers in *Paramecium*. For example, when a few drops of this diluted porphyrin were added to cultures of *Paramecium* and the cultures then exposed to sunlight all the organisms were killed within 10 minutes, whereas control cultures with the same amount of porphyrin, when left in the dark, remained unaffected.

In our experiments we have grown cultures of Tetrahymena in the presence and absence of light. When cultures were grown on agar for 4 days in continuous light, growth was diminished and no pigment appeared. When given 12 hours light and 12 hours dark, growth was better and no red pigment appeared. However, when the organisms were grown in the dark growth was best and the pigment was abundant. Cultures containing organisms with red pigment were exposed to continuous or intermittent light. After a few days no red organisms could be found but only red pigment in the medium. In the control cultures kept in the dark, organisms containing the red pigment were still abundant. It seems that absence of light favors growth of organisms with red pigment.

2. *Isolation and identification of the pigment from Tetrahymena.* Reddish and red-brown materials which had been obtained from Tetrahymena grown in test tubes, as described above, were examined and as far as could be determined the color of these small amounts of pigment appeared to be due to

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protoporphyrin. To obtain more of the pigment, Tetrahymena was grown in 3 Blake flasks. Each flask contained one liter of the agar medium described above supplemented with 0.1% Bacto yeast extract. After 10 days growth in the dark, the agar surface appeared to be somewhat brownish in color. The surface of the agar was washed with water and the suspension which was obtained was centrifuged. Two layers separated out at the bottom of the centrifuge tube, a thick white lower layer containing the cells, and a thinner red-brown upper layer containing pale pinkish spherical bodies 3-4 μ in diameter evidently derived from the cells. The supernate which contained no porphyrin was discarded.

The upper red-brown layer was separated from the lower layer by gently suspending it in a small amount of acetone and decanting it. The red-brown suspension was extracted first with acetone and then several times with 10 cc volumes of acetone-HCl 100:1 (v/v). The acetone-HCl solution was transferred to ether and fractionated by extracting successively with increasing concentrations of aqueous HCl. Two porphyrin fractions were obtained. Most of the porphyrin was extractable with 0.5-1.0 N HCl which suggested that this was protoporphyrin. About one tenth of the total porphyrin could not be extracted from ether solution with 1 N HCl although it appeared to have the absorption spectrum of protoporphyrin. This latter porphyrin became extractable only when the acidity was increased to 8 N HCl. However, once the porphyrin became soluble in the aqueous phase it came to have the HCl number and absorption spectrum of protoporphyrin. From these properties it may be inferred that a fraction of the total protoporphyrin in Tetrahymena is bound to some fatty material perhaps in ester linkage. Treatment with 8 N HCl appears to split this linkage.

A study of the absorption spectra of the 2 porphyrin fractions supported the identification of this porphyrin as protoporphyrin. With a Cary spectrophotometer determinations were made on the porphyrin, of the positions of the absorption maxima in ether solution and the ratios of the absorption maxima to each other. The positions of the maxima and the ratios agreed with those found for crystalline protoporphyrin. From the density of absorption it is estimated that a total of 0.6 mg of protoporphyrin was obtained from the Tetrahymena culture of the 3 Blake flasks. By a paper chromatography method further support was obtained for the identification of the porphyrin as protoporphyrin.

3. *Discussion.* Free protoporphyrin in contrast to its iron complex, heme, has been reported to occur normally in relatively large amounts only in the Harderian gland of the genus *Mus*. In all other instances, protoporphyrin accumulates due to some unknown metabolic lesion, as in the case of a mutant *Chlorella*(5,6) or in porphyria cutanea tarda of humans(7). It is interesting to note that in the case of the *Chlorella* mutant as well as in the present *Tetrahymena geleii* W strain, protoporphyrin is formed most readily on solid media in the dark and generally in older cultures. Heme is present in Tetrahymena as determined by the pyridine hemochromogen test so that at least qualitatively iron can be inserted into the protoporphyrin ring by the organism. The finding of the red pigment outside the cells suggests that the cells containing the pigment may excrete it or may break open more readily than cells not containing the pigment.

4. *Summary.* A strain of *Tetrahymena geleii* W at the end of its rapid phase of growth on solid media in darkness turns red-brown. The main pigment component has been identified as protoporphyrin. A minor component containing protoporphyrin appears to be bound to some fatty material.

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