Observations on *Phaeodactylum tricornutum*

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SUMMARY: *Phaeodactylum tricornutum* Bohlin was isolated from a marine tank at Woods Hole, Mass., U.S.A. It appeared to be identical with the Plymouth strain of '*Nitzschia closterium* forma *minutissima*'. Cells were of two characteristic types, oval and fusiform, each of which remained constant for many cell divisions in clonal culture. Triradiate cells arose rarely as atypical forms of the fusiform variety. Oval cells could arise as endospores within a fusiform cell. The transition from oval to fusiform was also studied, but details of the life cycle remain to be worked out. Electron micrographs showed the fusiform cells to be devoid of any organized siliceous structure, in agreement with previous observations. However, the oval cells were seen to possess a silica valve of a pennate diatom type, resembling those of the genus *Cymbella*. Only one valve was present on each cell, the remainder of the cell wall being unsilicified. The valve was 6.2μ . long, was equipped with a raphe, and was perforated by pores arranged in 60 striae. Oval and fusiform cells both contained approximately the same amount of silica (0.4–0.5 % dry weight). In each case, most of this silica could be recovered as a particulate fraction resistant to digestion in hot nitric acid. The silica obtained from oval cells was in the form of diatom valves, whereas that from fusiform cells consisted of irregular particles clearly not derived from broken silica walls.

Mucilaginous capsular material, soluble in hot water, represented 16 % of the dry weight of oval cells; it was absent from fusiform cells. Acid hydrolysis and paper chromatography indicated xylose, mannose, fucose, and galactose as components of the capsule.

Allen & Nelson (1910) isolated a small marine organism, which they referred to as '*Nitzschia closterium* W. Sm. forma *minutissima*'; unfortunately they did not publish any formal description or illustration of it. The fusiform cells resembled in shape those of the diatom *N. closterium* (Ehrenberg) W. Sm., but the dimensions were smaller. Cultures of this smaller organism isolated by Allen & Nelson (1910) have been maintained at Plymouth Marine Laboratory, Devon, England, and distributed to laboratories around the world. Since it grows rapidly in a wide range of temperature and illumination and in many different nutrient media, much of the fundamental work published on marine diatoms has been carried out with this strain. In addition, it has been extensively used as food for raising a variety of marine animals. Some unusual features exhibited by this organism have prompted further studies to elucidate its nature and affinities.

Barker (1935) observed that a contaminated culture of the Plymouth strain, which had been maintained at the Hopkins Marine Station in California for several years, contained spindle-shaped cells, oval-shaped cells, and cells shaped like three-pointed stars. In the course of isolating bacteria-free cultures, he observed a tendency for the spindle-shaped cells to become less numerous

on successive transfers to fresh agar while the number of oval cells increased. When grown in liquid media, on the other hand, a gradual reversion to spindle-shaped cells occurred. Triradiate cells were only rarely observed. Barker confirmed that these various cell forms all belonged to a genetically pure line. In 1942 Wilson & Lucas reported that triradiate forms of this organism arose independently in cultures maintained in Plymouth and in Hull. In a detailed illustrated account of this unusual organism, Wilson (1946) reported the presence of fusiform, triradiate and cruciform cells, and observed that both fusiform and triradiate cells sometimes gave rise to ovals. The ovals so formed either divided to produce more ovals or grew two or three arms to form fusiform or triradiate cells, respectively. Unlike the other cell types, the oval cells tended to produce mucilaginous capsules. Wilson did not observe auxospores, and stated that they were therefore not necessary for the elongation of the cells. The cell walls were so weakly silicified that he could not distinguish the valve markings characteristic of the Nitzschioideae. He considered the triradiate form to be identical with an organism of doubtful affinities, *Phaeodactylum tricornutum* Bohlin, 1897. Electron micrographs of fusiform and triradiate cells of the Plymouth strain showed no evidence of a characteristic diatom structure in the walls (Hendey, 1954; Bourrelly & Dragesco, 1955). In the material examined by these workers there were evidently few or no oval forms present, since these were not illustrated. In the course of physiological studies of the factors responsible for the changes of form of this organism, we prepared electron micrographs of oval as well as of fusiform cells. It immediately became apparent that *P. tricornutum* is an even more unusual and interesting organism than we had hitherto suspected. The structure of the walls of oval cells, as well as chemical and physiological differences between oval and fusiform cells, are described in this paper.

METHODS

In September 1956, we observed fusiform cells of *Phaeodactylum tricornutum* in a large outdoor tank behind the Woods Hole Oceanographic Institution, containing sea water enriched with a commercial fertilizer. By serial streaking of the cells on enriched sea-water agar plates we isolated the organism in bacteria-free culture. We also obtained from Dr M. Droop (Marine Station, Millport, Scotland) two other pure cultures of *P. tricornutum* for comparison: Millport No. 14, isolated by Droop in 1951 from a rock pool on the island of Segelskär, off the south coast of Finland, and Millport No. 15, the Plymouth strain. When the Woods Hole strain and the two European strains were grown in separate samples of the same batch of medium, they appeared identical. For the studies reported in this paper we used only the Woods Hole strain.

Two basal culture media were used:

(1) Enriched sea water, which consisted of nine volumes of sea water to one volume of a supplementary mineral solution; the final concentrations of added minerals were $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 0.01 %; K_2HPO_4 , 0.002 %; 0.5 p.p.m. Fe; 0.3 p.p.m. Zn; 0.1 p.p.m. each of B, Co, Cu, Mn, Mo; $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$ to give a

Si concentration of 5 p.p.m. In some cases Tryptone (Difco) was added at a concentration of 0.1 %.

(2) Artificial sea water, which consisted of NaCl, 2.0 %; $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 0.05 %; K_2HPO_4 , 0.01 %; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 %; trace elements and silicate as in (1). For solid media, agar was employed at a concentration of 1.0 % (w/v). The cultures were constantly illuminated with white fluorescent lights (300 f.c.) and maintained at 21°. Aeration of liquid mass cultures was provided by a slow passage of air filtered through sterile cotton wool.

Samples of dry organisms were analyzed for major constituents as follows: (1) Micro-Kjeldahl N determinations ($\times 6.25 = \text{protein}$); (2) total ash and total silica; (3) silica in valves after digestion with hot concentrated nitric acid. In addition 100 mg. samples of dry organisms were extracted by: (1) successive methanol extractions; (2) cold dilute hydrochloric acid, pH 3; (3) water at 100°. Ash and silica were determined in the cell residues remaining after these extractions. The methanol extracts (1) were pooled, the solvent removed by evaporation, and the solid residue, comprising various pigments and lipids, was dried in a vacuum desiccator and weighed. The cold aqueous extract (2) contained inorganic salts and soluble carbohydrates including leucosin. Free and combined hexoses were determined quantitatively by the anthrone reaction and were expressed as glucose equivalents. Two successive water extractions at 100° (3) removed the capsular material from the cells. The extracts were concentrated to 5 ml. and poured into 15 ml. ethanol. The flocculum which formed was removed by centrifugation, washed, dried and weighed.

Paper chromatographic analysis of the capsular material was carried out as follows. A sample of the dried material (5 mg.) was heated in a sealed tube with 1.0 ml. of 0.7 % H_2SO_4 for 6 hr. at 105°. The hydrolysate was neutralized with BaCO_3 , and the supernatant fluid evaporated to dryness and redissolved in one drop of water. This extract was chromatographed on paper for 16 hr. together with reference sugars, using either of two separate solvent mixtures: (a) butanol: H_2O :acetic (4:1:1), or (b) phenol: H_2O (4:1) in ammonia vapour. The chromatograms were dried, sprayed with 1 % (w/v) aniline phthalate, and developed for 5 min. at 100°.

Silicon determinations were carried out as follows. Material such as acid-cleaned valves, or the ash from dried cells or extracted cell residues, was fused with excess sodium carbonate in platinum crucibles. The flux was dissolved in water, and the silicon in solution was determined colorimetrically by the ammonium molybdate reaction (Lewin, 1954).

In preparing material for electron microscope observations we used whole cells, walls from cells burst by rapid transfer into distilled water, and silica valves prepared by digesting the cells with hot concentrated nitric acid. An R.C.A. Model EMU electron microscope was used.

RESULTS

Character of colonies on an agar surface

The colonies of *Phaeodactylum tricornutum* which formed from cells streaked on agar were mainly of two different types. Those composed of fusiform cells were dark brown, rounded, with smooth margins, and showed a satiny iridescence due to the parallel alignment of the cells. Colonies of oval cells were more diffuse, with irregular edges where individual cells migrated over the surrounding agar, and of a lighter shade of brown since the cells were separated from one another by colourless mucilaginous capsules. These two types of colonies could be readily distinguished with a binocular dissecting microscope, and, after growth for 2 or 3 weeks, even with the naked eye. It was possible also to recognize colonies, initially comprising only fusiform cells, but later giving rise to oval cells, which began to spread over the agar from one or more sites at the edge of the colony. The fact that oval cells could creep over a surface in this fashion, as do many species of pennate diatoms, suggested that cells of this type might possess a raphe, as do all motile diatoms.

Observations on the life cycle

Wilson (1946) attempted to follow the life cycle of his organism by examination of cells from liquid cultures. In cultures originating from oval cells, he noted that cells with short arms appeared, and that the average length of the arms increased in successive transfers. Although normally such cells bore two arms, he occasionally observed the origin of triradiate or other variant forms. We followed the development of individual cells on agar by drawing them at daily intervals, and observed:

- (a) Oval cells divided to form oval cells (confirming Wilson's observations).
- (b) Fusiform cells divided to form fusiform cells of similar length and form (confirming Wilson's observations).
- (c) Inflated fusiform cells, approximately 25μ . long, produced a pair of ovals (cf. Wilson, Fig. 2) by division of the protoplasm within the central enlarged region of the cell. These oval cells sometimes divided again and were ultimately released by rupture of the parent wall. This observation did not bear out Wilson's supposition that oval cells arose from fusiform cells by unequal division.

Liquid and agar cultures also occasionally contained spherical auxospores, naked vacuolate protoplasts $6\text{--}12\mu$. in diameter, which were usually attached to an empty wall of a fusiform cell. All our attempts to follow the fate of individual auxospores on agar have so far proved unsuccessful.

Among 64 subclones which originated from single colonies on agar and were grown in liquid medium, one was found in which the cells were initially all triradiate. Since this form was believed to be atypical, the following experiment was carried out. The culture was allowed to age until abundant oval cells had been produced. It was then streaked on agar, and after 10 days 100 colonies of oval cells were isolated and transferred into liquid medium 1. When examined after 28 days, these cultures contained only oval cells.

Transfers were then made into medium 1 + Tryptone, which had been found to promote the production of armed cells. Ten days later all the cultures had produced abundant fusiform cells. Since no triradiate forms were seen, this form evidently does not persist beyond the intermediate stage of the oval cells. This confirms observations by Wilson (1946) and by Droop (Hendey, 1954), and supports the view that triradiates are not a normal stage of the life cycle. These studies are being continued with a view to determining what factors may be responsible for the changes in cell form and whether a sexual cycle may be involved.

Cell walls

The oval cells of *Phaeodactylum tricornutum* are approximately 8μ . long and 3μ . wide; the fusiform cells extend to a length of 35μ . Intact oval and fusiform cells, and cell membranes of both cell types prepared by bursting the cells in distilled water, were examined with the electron microscope. No perforated valves were seen on any of the intact fusiform cells (Pl. 1, figs. 1, 2), nor on the fusiform membranes (Pl. 1, figs. 3, 4), confirming the observations of Hendey (1954) and of Bourrelly & Dragesco (1955). However, the oval cells were seen to possess a valve of opaque material, presumably silica, of a typical pennate diatom structure (Pl. 2, figs. 5, 7). This valve was more clearly distinguishable in preparations of cell membranes freed from other cell material, and appeared as a silicified area on one side (Pl. 2, figs. 6, 8).

A typical diatom cell regularly possesses a silica frustule, composed of two valves and connecting bands, which completely encloses it in a silica shell. In both cell types of *Phaeodactylum tricornutum* the cell membranes appeared to be composed of a pair of overlapping parts, most readily perceived at the ends of cells seen in side view (Pl. 1, fig. 2 and Pl. 2, fig. 9). These evidently correspond to the epitheca and hypotheca of a typical diatom. The unsilicified portions of the cell membranes were transparent to the electron beam, pliable and structureless, except for faint indications of a fibrous nature. A unique feature of the oval cells of *P. tricornutum* was the presence of only one valve covering only part of the cell surface (Pl. 2, fig. 9). This was the case in over 90 % of the cells examined; in the remainder, for one reason or another, no valve could be seen.

When oval cells were digested with nitric acid, the cell contents and membranes disappeared and only the valves remained, thereby confirming their siliceous nature. The 'cleaned' valves were c. 6.2μ . long by 1.6μ . wide, with a raphe, a central and polar nodules, and 60 rows of pores or striae (equivalent to 95 in 10μ .). The apical axis of the valve was slightly curved producing a shape characteristic of the genus *Cymbella* (Pl. 2, fig. 10). Identical silica valves were also found in the two *Phaeodactylum* strains sent to us by Droop. Though the valve markings of the strain used here are so fine that it would be impossible to distinguish them with a light microscope, we considered that the valves themselves should be detectable without recourse to electron microscopy. Once we knew what to look for, we extracted the pigment from some oval cells with methanol, stained the cells with toluidine blue, and

mounted them in Hyrax. When we examined them under oil immersion we could make out in some cells the dark line of the axial area on the surface.

Chemical analyses of fusiform and oval cells

By inoculating tubes of liquid medium with oval and with fusiform cells from selected colonies of *Phaeodactylum tricornutum* on agar, clones of the two cell forms were isolated. Mass cultures of each were grown in 2 l. Erlenmeyer flasks under conditions of constant temperature, light, and aeration. After incubation for 16 days, aeration was stopped. The oval cells, which grew as clumps, settled within a few minutes to the bottom of the flask, whereas the fusiform cells remained in suspension for some hours. The cells were concentrated and separated by centrifugation. The fusiform cells packed to form a smooth pasty mass, occupying only half the volume taken up by the gelatinous mass of oval cells. Microscopic examination revealed among the fusiform cells less than 1 % of ovals. The yields from 1.5 l. culture medium in one experiment are summarized in Table 1.

Table 1. *Yield of Phaeodactylum tricornutum from 1.5 l. of culture medium*

	Oval cells	Fusiform cells
Packed cell volume (ml.)	5.8	2.7
Total number of cells	2.15×10^{10}	2.17×10^{10}
Dry weight (mg.)	481	439

Table 2. *Chemical composition of oval and fusiform cells of Phaeodactylum tricornutum*

	Organisms	
	Oval	Fusiform
	% of dry wt.*	
Lipid	24	34
Protein	34	41
Carbohydrate		
Soluble in cold dilute acid	3	2
Soluble in hot water, precipitated with ethanol (capsular material)	16	0
Miscellaneous		
Soluble in cold dilute acid (not carbohydrate, not ash)	10	10
Soluble in hot water, not precipitated with ethanol	3	2
Ash	13	12
Total	103	101

* Averages of 2-3 determinations

The cells were analyzed as described on p. 420 (Methods), and the results shown in Table 2. Approximately 16 % of the dry weight of oval cells consisted of a mucilaginous material, soluble in hot water, and corresponding to the cell capsules which stain metachromatically with toluidine blue. Paper chromatograms of the hydrolysate of this substance indicated the presence of

xylose, mannose, fucose and galactose. A mucilaginous fraction was not present in the fusiform cells, which lacked capsules, but which contained higher percentages of lipid and protein than the ovals.

Silica determinations were carried out on samples of dried cells of each type which had been rinsed with cold dilute acid, washed, dried and ashed. Both oval and fusiform cells contained approximately the same amount of silica (0.4–0.5 % of dry weight). This was unexpected, since no silica walls have ever been observed in the fusiform cells. Separate samples of oval and fusiform cells were digested with hot concentrated nitric acid. After this treatment, a small amount of white flocculent material remained floating on the surface of the nitric acid in each case. No silica valves were present in this flocculum. In the case of the oval cells, there also always remained a white precipitated deposit, which proved on chemical analysis to contain most of the cell silica (0.3–0.4 % of dry weight), and which consisted of diatom valves as revealed by the electron microscope. In two out of four analyses of fusiform cells, a white precipitate was also obtained after nitric acid digestion. This likewise proved to contain most of the cell silica (0.3–0.4 % of dry weight), but when examined with the electron microscope it appeared to be composed only of structureless debris, comprising irregular particles of various sizes, plainly not derived from silica walls.

DISCUSSION

It seems to be the opinion of most workers who have studied *Phaeodactylum tricornutum* that the oval and the fusiform cells are the typical prevalent stages in the life cycle. With prolonged cultivation on an agar surface the oval cells predominate, apparently being favoured since they have some ability to spread over the agar surface. Barker (1935) and Droop (see Hendey, 1954) have observed the transition to a predominance of oval cells on solid media. On the other hand, on prolonged cultivation in a liquid culture the fusiform cells predominate. Apparently they are at an advantage since in an actively growing culture they are more buoyant, whereas the oval cells tend to associate in clumps and sink to the bottom. Both Barker and Droop have reported a tendency for the formation of fusiform cells in liquid culture.

The triradiate cells have been the only form hitherto readily recognizable in samples of littoral water from nature. This is evidently because the fusiform cells might be mistaken for shortened *Nitzschia closterium*, and the ovals cells, when noticed at all, would be classified as minute, unidentifiable pennate diatoms. Triradiate forms originate rarely in the laboratory, but under certain conditions, as in static cultures, such cells may be favoured by selection, perhaps because of a decreased rate of sinking, and may overgrow the other forms. In addition to triradiates, cruciform cells (Wilson, 1946), curved cells, and other abnormal forms have occasionally been observed. When such a morphological abnormality arises, it reproduces clonally, forming daughter cells of the same shape as the parent cell, since cell division takes place in a longitudinal plane. The cell membrane is evidently inelastic enough

to maintain its characteristic shape through successive cell divisions. However, variants of this sort tend to persist only until they produce oval cells, since these in due course typically give rise to normal fusiform cells.

Some data on the chemical composition of '*Nitzschia closterium* forma *minutissima*' have been reported by previous workers. Bidwell, Krotkov & Reed (1952) analyzed for free and bound sugars in cells of the triradiate variety. They found approximately 0.3 mg. ethanol-soluble sugars (glucose, fructose, sucrose) in 10^{10} cells. According to our calculations these free sugars would represent approximately 0.15 % of the dry weight. These workers also reported 6.4 mg. carbohydrate from the ethanol-insoluble residue, which we estimate would represent approximately 3.2 % of the dry weight. After acid hydrolysis, glucose, representing 2.5 % of the dry weight, was found to be the main constituent of this fraction. This glucose most probably came from leucosin, a water-soluble polymer of glucose characteristic of the Chrysophyta and reported from *Phaeodactylum tricornutum* by Parke (Hendey, 1954). In our analyses, leucosin could account for the 2-3 % of soluble carbohydrate in the cold dilute acid extract, a value in agreement with that calculated from the data of Bidwell, Krotkov & Reed (1952). Probably there were few or no oval cells in the material analyzed by these workers, since they did not report insoluble polysaccharide sheaths. Ketchum & Redfield (1949), using an empirical formula, calculated the proportions of protein, carbohydrate and lipid in the same organism, based on the R value (degree of reduction) and on the percent protein. Their figures indicated 42 % protein, 25 % lipid, 33 % carbohydrate in the organic fraction, and an ash residue representing 19.5 % of the dry weight. Expressed on the same bases, our values were, respectively, 39, 28, 22 and 13 % for ovals, and 47, 39, 2 and 12 % for fusiform cells.

Harvey (1955) reported that about 1 % of the dry weight of the *Phaeodactylum tricornutum* he examined was silica. In the present study, the washed cells of both forms of our *P. tricornutum* were found to contain approximately 0.4 % silica on a dry-weight basis. This is present in the oval cells largely as silica valves. It is not known in what chemical condition the silica exists in the fusiform cells, since no recognizable structures remained after digestion with concentrated nitric acid. In comparison with other diatoms, *Phaeodactylum* is very weakly silicified, and enough silicon dissolves from Pyrex glass vessels in alkaline culture media to fulfil its meagre requirements. Harvey (1955) reported that heavy crops of *P. tricornutum* were produced in enriched sea water in hard-glass vessels without added silicate or silica sol, and we observed no difference in the rate of growth of oval or fusiform cells in artificial sea-water media, whether silicon was added or not. Analyses and physiological studies of this organism have been extensively quoted as representative of the diatoms. Present evidence seems to indicate that *P. tricornutum* is a most atypical diatom; its taxonomic position will be discussed in the following paper (Lewin, 1958).

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EXPLANATION OF PLATES

PLATE 1

Electron micrographs of *Phaeodactylum tricornutum*. Each scale line represents 2 μ .

- Fig. 1. Fusiform cell.
 Fig. 2. Fusiform cells.
 Fig. 3. Membrane from fusiform cell.
 Fig. 4. Membranes from fusiform cells.

PLATE 2

Electron micrographs. Each scale line represents 2 μ .

- Fig. 5. Oval cells.
 Fig. 6. Membranes from oval cells.
 Fig. 7. Oval cell.
 Fig. 8. Membrane from oval cell.
 Fig. 9. Oval cell, side view.
 Fig. 10. Silica valves of oval cells.

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J. C. LEWIN, R. A. LEWIN AND D. E. PHILPOTT—*Phaeodactylum tricornutum*.
PLATE 1

(Facing p. 426)

J. C. LEWIN, R. A. LEWIN AND D. E. PHILPOTT—*Phaeodactylum tricornutum*.
PLATE 2