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Morphogenesis and Polymorphism of *Ankistrodesmus* spp.

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SUMMARY: Pure cultures of *Ankistrodesmus* spp. showed continual morphological fluctuation when growing in complex organic media, and to a lesser (though detectable) extent in mineral media. Clonal populations were no less variable. In mineral media, variation of *A. braunii* was associated with the stationary phase of growth only, but in the complex organic test medium was seen in logarithmic phase as well. The normal processes governing cell morphogenesis are described for *A. braunii*. Their normal sequence is disrupted by increasing osmotic pressure of the medium in reverse of their normal order in time and of their hypothetical serial coupling to the metabolism concerned with cell growth. The final uncoupling, that of growth from initiation of cell division, is also accomplished by a wide range of C–N heterocyclic ring structures in very low concentration, which may indicate that nucleic acid metabolism is the system concerned at this point in the sequence.

During the course of a search for organic growth factors for algae of diverse taxonomic position, a strain of *Selenastrum* sp. was found to produce large numbers of morphologically variant cells when grown in the presence of small amounts of yeast extract in mineral medium. Variation was nearly as marked in a complete defined *Lactobacillus* medium containing nineteen amino acids and various other growth factors and metabolites (Table 1) even at relatively low concentrations.

Agardh (1820) introduced the term ‘polymorphism’ to describe the occurrence of morphological variants of a given form which simulate other related or unrelated species. Artari (1892) and Klebs (1896) included excellent critical reviews of the many observations of the phenomenon made during the nineteenth century. Various workers of the present century, beginning with Beijerinck (1904), attempted to apply the pure-culture methods of bacteriology in the study of algal polymorphism. The group of phycologists at the University of Geneva, headed by Chodat, was most active in this field. However, in spite of both Klebs’s and Artari’s recommendations for study of morphological changes in individual organisms in carefully controlled microcultures, the Geneva workers failed to clarify the essential question of inherent variability in clonal lines by investigating mass cultures at the time of maximal growth or later. Chodat (1913) at first interpreted his studies as proving glucose to be the direct cause of polymorphism, and on the basis of behaviour in glucose media attempted the classification of many ‘elementary species’ of the order Chlorococcales, some of which are still recognized by present-day

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taxonomists. Hofmann-Grobéty's (1912) results may now be interpreted as demonstrating that the high toxicity of glucose for *Raphidium minutum* Naeg. (*Selenastrum minutum* (Naeg.) Collins) permits survival of only rare individuals which are probably resistant mutants. Vischer (1920) considered the aberrant individuals in his cultures of *Ankistrodesmus braunii* to be due to inhibition of cytokinesis by hexoses. Both these investigators found peptone to be protective against toxicity of glucose, though peptone itself was highly toxic when used alone in low concentrations.

The possibility of genetic causes of this commonly observed morphological variability of the Chlorococcales was not taken seriously at first by Chodat. He, like other nineteenth-century workers thought of 'mutations' as sudden phenotypic bursts of new, complete species. Oettli (1927) concluded that the morphological fluctuation he observed in five 'elementary species' of *Ankistrodesmus* was due to 'simple, non-hereditary modifications', but admitted he had not attempted to prove his notion, even when a population of elongate individuals appeared to arise from one of spherical individuals upon transfer to different media. Chodat later revised his thinking (1929) to propose step-wise 'generalized micromutation' of single characters as the explanation of repeatedly occurring pigment variants in *Chlorella rubescens*. However, up to his death in 1934, he had made no attempt to explain on this basis the nature of variation in cell-shape, nor to carry out the microculture studies urged by his critics.

The independent discovery of polymorphism in *Selenastrum* sp. growing in a defined organic medium suggested that an extensive re-investigation of the phenomenon in the light of modern biochemistry and genetics might disclose the extent of morphological variability to be expected in cultured strains of the simpler green algae, as well as some of the actual mechanisms of cellular morphogenesis. Further, low concentrations of common organic metabolites in solution should simulate natural ecological conditions, such as those in a pond in late summer, and elucidate the extent to which such factors might induce significant variations in wild populations of planktonic algae. Such naturally-occurring 'phenocopies' might well cause taxonomic confusion, and probably have done so in the past, whenever identification has been based on a few organisms found in a collection rather than on cultures isolated from the sample.

METHODS

Mass populations

Experiments were designed to test the effect of the defined organic medium (Table 1) and its fractional variants (Table 2) on stationary-phase cultures of mass populations; i.e. under conditions comparable to those of the Geneva studies. Only experiments with *Ankistrodesmus* spp. will be reported at the present time. Studies of *Selenastrum* spp. and *Scenedesmus* spp. were also begun, but were not extended beyond the first mass culture runs, as these organisms grew less rapidly and were more difficult to handle in centrifuging, dilution, etc., than *Ankistrodesmus* spp.

Table 1. *Composition of the complete defined organic medium used*

Constituent	Quantity/ 100 ml.	Constituent	Quantity/ 100 ml.
DL-Alanine	0.8 mg.	L-Tyrosine	0.2 mg.
L-Arginine	0.2 mg.	DL-Valine	1.0 mg.
L-Aspartic acid	0.6 mg.	Adenine sulphate	0.02 mg.
L-Cysteine	0.8 mg.	Guanine hydrochloride	0.02 mg.
L-Cystine	0.8 mg.	Uracil	0.02 mg.
L-Glutamic acid	1.0 mg.	Thiamine hydrochloride	0.4 µg.
L-Glycine	0.2 mg.	Riboflavin	0.4 µg.
DL-Histidine	0.4 mg.	Nicotinic acid	0.4 µg.
DL-Isoleucine	1.0 mg.	Biotin	0.008 µg.
DL-Leucine	1.0 mg.	Pyridoxine hydrochloride	0.8 µg.
L-Lysine	0.4 mg.	p-Aminobenzoic acid	0.08 µg.
DL-Methionine	0.8 mg.	Folic acid	0.016 µg.
DL-Phenylalanine	0.4 mg.	Calcium pantothenate	0.4 µg.
L-Proline	0.2 mg.	Glucose	0.04 g.
DL-Serine	0.4 mg.	Sodium acetate	0.024 g.
DL-Threonine	0.4 mg.	GFS* solution	To make
DL-Tryptophane	0.4 mg.		100 ml.

* GFS, see Gerloff, Fitzgerald & Skoog, 1950.

Table 2. *Code for types of media*

Medium no.	Constituents
1 ±	Lacking or having only aa group 1 (monoamino monocarboxylic): gly., ala., leu., isol.
2 ±	Lacking or having only aa group 2 (monoamino dicarboxylic): asp., glut.
3 ±	Lacking or having only aa group 3 (diamino monocarboxylic): arg., lys.
4 ±	Lacking or having only aa group 4 (S-containing): cysti., cyste., meth.
5 ±	Lacking or having only aa group 5 (Ar): φala, tyr.
6 ±	Lacking or having only aa group 6 (heterocyclic): hist., pro., tryp.
7 ±	Lacking or having only vitamins: biotin, nicotinic acid, riboflavin, thiamine HCl, pyridoxine HCl, PABA, folic acid, Ca pantothenate
8 ±	Lacking N bases: adenine, guanine, uracil
9	Complete
10	Complete—glucose (i.e. + acetate alone)
11	Complete—acetate (i.e. + glucose alone)
12	Complete—acetate and glucose
13	GFS* alone
14	13 + yeast extract
15	13 + glucose
16	13 + acetate
17	13 + acetate and glucose
18	13 + all amino acids
19	Complete—all amino acids

* GFS, see Gerloff, Fitzgerald & Skoog, 1950.

Ankistrodesmus falcatus v. *acicularis* 101 and *A. falcatus* 188, both from the Indiana University Algal Culture Collection, Bloomington, Indiana, U.S.A., were the first test strains. In these and all subsequent experiments, culture vessels were glass-capped rather than plugged with cotton-wool, and all experimental cultures were run in duplicate. In the first run, suspensions of organisms from the morphologically most homogeneous stock cultures, as

determined by quantitative count, were inoculated into the liquid test media and incubated for 8 weeks at 24° with continuous daylight fluorescent illumination of approximately 400 f.c. At the end of this time the suspended sediment from the experimental cultures was sampled and counted differentially. Long-term effects were studied by retaining all cultures from this run for a total of 150 days; while at 80 days cultures growing on media nos. 9, 12, 13 and 14 (of Table 2) were subcultured into the same media and the daughter cultures incubated for the remainder of the experimental period. Cultures which showed impressive changes from their earlier state or from their parent cultures on preliminary examination were differentially counted at the end of the 150-day period. Finally, cultures which had been selected for this count were inoculated into the test media and their daughter cultures counted at 21 days. 'GFS', or medium no. 13 of Table 2, which was the Gerloff, Fitzgerald & Skoog (1950) version of Chu's (1942) medium no. 10, and medium no. 14 (Difco yeast extract in a concentration of 0.1 g./100 ml. of GFS) were used as control media for all experiments.

Clonal isolates

Results of the experiments with mass populations pointed out the wisdom of Artari (1892) and Klebs (1896) in urging the study of variants as they arose from single organisms. The establishment of known clonal lines might rule out a process of diploidization, segregation and recombination if it were involved in the morphological variation.

Some technical difficulties arose in the course of attempts to use the more common isolation procedures, which revealed certain characteristics of these organisms. *Ankistrodesmus* showed very low viability when spread on agar plates. Later experiments with the micromanipulator confirmed the impression that spreading techniques were lethal because of the extreme fragility of the organisms during mechanical handling. The technique finally evolved for successful isolation of single organisms with the de Fonbrune micromanipulator is described elsewhere (McMillan, 1955). By this method clonal substrains were isolated from each of the strains used in the earlier experiments, as well as a strain of *Ankistrodesmus braunii*. The latter species was added to the study at this time in order to extend observations within the genus, and because of the detailed studies of polymorphism in this species by Oettli (1927), Vischer (1920) and Chodat & Malinesco (1893).

Each of the clones was inoculated in triplicate into GFS medium as soon as turbidity was grossly visible in the isolation tube, and the transfers examined at 22 days. The clone of *Ankistrodesmus braunii* was representative of the degree of variation found in all the cultures, and so was the only one counted quantitatively.

Microcultures

The continual morphological variance seen in the clonal cultures necessitated detailed analysis of the phenomenon from as many individual organisms as possible. Since micromanipulative isolation of large numbers of individuals was tedious and time-consuming, a method of microculturing of relatively

large numbers of organisms was sought. The Levy haemocytometer chamber proved ideal for this purpose. Organisms from the desired source were prepared and diluted with culture medium to give a final concentration not greater than $5-6 \times 10^4$ organisms/ml. The suspension was introduced with a Pasteur pipette into the haemocytometer, which had previously been sterilized and fitted with a sterile no. 1 coverslip held in place by a film of sterile white vaseline along either edge. The chamber was completely filled, including the moats, and sealed with melted sterile vaseline except for a small space at one end of the transverse moat, which was left open to permit exchange of gases. The location of individuals was plotted on a map of the chamber at zero time, and their subsequent development followed at intervals as desired. Inoculated chambers were incubated in large moist chambers fitted with small beakers of sterile water containing Kleenex wicks, at the same illumination and temperature as used in earlier experiments. The tube used for inoculation of the microculture was routinely retained and incubated as a control. By this means, detailed observations were made of thousands of individuals in various media, and the development of their progeny followed for as long as two to three months. Photographs were made of organisms *in situ* or from the control tube cultures, on Kodachrome, Microfile, Adox KB-14 and Kodak Plus-X films, by using the Leica Micro-Ibso apparatus. AO $\times 45$ achromatic and apochromatic and $\times 90$ apochromatic objectives and $\times 10$ and $\times 15$ compensating oculars were used.

*Normal development of Ankistrodesmus braunii
under photoautotrophic conditions*

An intensive study was made of normal development of organisms in GFS medium. Ages of inoculum varied from 42 hr. to 85 days. Organisms which were initially 'atypical' as well as 'typical' were followed throughout the entire growth cycle.

Effect of specific media upon development of log-phase cells

Carbohydrates. Since Chodat (1913) had indicted glucose as the cause of polymorphism, and our earlier experiments implicated the complete defined medium or some of its components, these media were tested in microcultures. Runs were set up in medium no. 9 (the complete medium), and glucose (medium no. 15) at 0.1–10.0 times its standard concentration in the complete medium (i.e. 0.04, 0.4, 2.0 and 4.0 %, w/v). The glucose media were made up in GFS (medium no. 13). Also, 4.0 % (w/v) glucose was made up in Difco yeast extract medium (medium no. 14) and in GFS medium containing 5 times the normal concentration of nitrate, as organic and inorganic N supplements, respectively, to test the further hypothesis of Chodat (1913, 1929) that an inadequate supply of N in the presence of glucose caused polymorphism. Controls for these experiments were both types of N supplement without glucose, as well as GFS medium. Fructose, mannose and galactose were tested at 4 % (w/v) to determine the degree of molecular specificity. Pentaerythritol in concentrations equimolar with the different glucose concen-

trations was used as a control for the effect of increased osmotic pressure, as suggested by Dr S. H. Hutner (personal communication). In addition to qualitative cytological observations in all these media, counts were made periodically in the glucose runs to determine the total population in each concentration in each chamber, and the total numbers of variants of all sorts.

Non-amino acid constituents of organic medium. These compounds were tested in tube culture only, as previous experiments had shown practical identity between tube and microcultures of the same age. All the substances listed in Table 1 with the exceptions of glucose and the amino acids were made up in GFS medium at 1.0, 2.5, 5.0 and 10.0 times their standard concentration in the complex medium. Samples from each tube were examined microscopically after 72 hr. growth under the usual conditions of illumination and temperature, for normally released progeny *versus* 'bulging' cells. All tubes were examined grossly for relative turbidity at 13 days, to ascertain whether adaptation had occurred in the course of several division cycles. Samples of approximately these ages, 72 hr. and 13 days, had proved to be definitive in earlier experiments, since 72 hr. is normally the middle of the log phase and 13 days is in the normal stationary phase.

Purines, pyrimidines and related compounds. Tube cultures were set up at different concentrations of the following compounds: imidazole, pyridine, bipyridine, pyridoxine hydrochloride, adenine, guanine, xanthine, cytosine, uracil, thymine and thiamine hydrochloride. All compounds were tested in duplicate tube cultures at concentrations of 10, 5, 2.5 and 1 $\mu\text{g./ml.}$, unless otherwise noted in Table 5. Control cultures were run in GFS medium. Samples from both experimental and control tubes were examined at 96 hr. and 280 hr. after incubation under the usual conditions.

RESULTS

Mass populations

Cell-types which were phenocopies of many recognized taxa of the Chlorococcales and other forms were found in all daughter cultures of the first group of experiments at one time or another and in the various media, in spite of the selection of inocula for maximum homogeneity and predominance of species-types. Many of the variants bore no resemblance to the parental types (Fig. 1). The distribution of these cell-types in representative experiments is shown in Figs. 2 and 3. It was evident that the morphological variability of *Ankistrodesmus* spp. (as well as of the other forms not included in this report) was as great as reported by the early workers, and sufficiently remarkable to warrant further study. The correlation of particular variant types with any given culture medium was not clear-cut. Rather than extensive attempts at such a correlation, either by statistical means or by repeated experiments of this type, an investigation of the basic biological causes of the variation was chosen.

In long-term studies of the same substrains, the proportion of variants to 'parental' species-types did not appear to be constant (Table 8). Attempts to

induce variant populations to revert to species-type by return to mineral medium were not uniformly successful; in fact, on three separate occasions (twice within one substrain) a variant became the predominant form and remained constantly so for some time. At present, it may be postulated that two independent mutations occurred in these strains, closely associated in time: one for modified cell shape (through alteration of metabolic pathways involved in cell elongation, cytokinesis, cell-wall formation, etc.) and one for metabolic efficiency. Unfortunately it has not been possible to test this hypothesis, for these strains were lost because of contamination, as were most of the substrains of the first experiments. However, this evidence of spontaneous alteration in populations is of considerable taxonomic interest. The clones of all three strains of *Ankistrodesmus*, when isolated, appeared fully as

Fig. 1. Cumulative record of all morphological types seen in all cultures of all strains* in the course of studies of mass populations. Since the average size of individuals varied considerably within each strain as well as between strains, these outline sketches are of various magnifications.

* Strain no. 1 = *Ankistrodesmus falcatus* v. *acicularis* 101 (Indiana University Algal Culture Collection).

2 = *Ankistrodesmus falcatus* 188 (Indiana University Algal Culture Collection).

3 = *Selenastrum* sp. 285 (Indiana University Algal Culture Collection).

4 = *Selenastrum* sp. 9 (McMillan isolate).

5 = *Scenedesmus dimorphus* 5 (McMillan isolate).

6 = *Scenedesmus quadricauda* 83 (Indiana University Algal Culture Collection).

7 = *Ankistrodesmus braunii* 245 (Indiana University Algal Culture Collection) (includes McMillan clonal isolate 245-St 1.1).

Key to occurrence of morphological types

Type no.	Species-type or originally predominant in strain no.	Predominance shifted to this type in strain no.	Seen occasionally in strains no.
1	1	2	All except 5
2	2	1	All except 5 and 6
3	7	—	All
4	—	—	All
5	—	1	All
6	—	—	All except 5 and 6
7	—	1	All
8	3 and 4	2	All except 5 and 6
9	—	1 and 2	All except 5
10	—	—	All
11	—	—	All
12	6 (species-type)	—	All except 1 and 2
13	5	—	All except 1 and 4
14	—	—	All except 2 and 3
15	—	—	3, 5 and 6
16	—	—	3, 5 and 6
17	—	—	All except 2 and 6
18	6 (original count)	—	All
19	6 (original count)	5	All
20	—	—	All
21	—	—	All
22	—	—	1, 2, 3 and 4
23	—	—	All
24	—	—	All except 5 and 6
25	—	—	2, 3, 4 and 5
26	—	1	1, 2, 3 and 7
27	—	—	All except 1
28	—	—	All except 1 and 2
29	—	—	2, 3 and 4
30	—	—	2, 3 and 4
31	—	—	4
32	—	—	3, 4
33	—	—	All except 5 and 6
34	—	—	2, 3 and 4
35	—	—	2, 3 and 4
36	—	—	2, 3, 4 and 7
38	—	—	4, 6
39	—	5 and 6	3, 7
40	—	—	5, 6
41	—	—	3, 5 and 6
42	—	—	All except 1
43	—	—	All except 1
44	—	—	All except 5 and 6
45	—	—	4
46	—	—	1, 2, 3 and 4
47	—	1	All except 5 and 6
48	—	—	1
49	—	—	1, 2
50	—	—	2, 3 and 4
51	—	—	2, 3 and 4
52	—	—	All except 1
53	—	—	7
54	—	—	7
55	—	—	All except 5 and 6

variable as the mass populations (Table 4). Most of the variant types were present in detectable numbers in the first transfer of each clone, though this variation was less marked in GFS medium than in organic media.

Fig. 2. Distribution of cell-types of Fig. 1, in representative runs of mass population studies of *Ankistrodesmus falcatus* v. *acicularis* 101 (Indiana University Algal Culture Collection). Inocula were prepared by pooling and centrifugally washing 3-week cultures. Cultures were differentially counted after 8 weeks of growth.

Phases of growth-cycle and variants

Two facts became evident during the course of the growth studies of *Ankistrodesmus braunii*. First, at the size inoculum chosen for the long-term studies, organisms were detectable at zero time and for a few hours thereafter; but there followed, in all cultures, a considerable lag period during which no growth was detectable, even under the low power of the dissecting microscope. Finally, about the sixth to tenth day, organisms were detectable and quantitative counts, discontinued after the first day, could be resumed (Fig. 4). The degree of decrease in direct count and length of lag phase varied inversely with the size of the inoculum (Fig. 5), except where organisms were concentrated by centrifugation and an inoculum larger than the normal stationary-phase density was used. The logarithmic phase of growth was characterized by several spurts, each followed by a macroscopic decrease in turbidity and marked decrease in the direct count by the microscope, together with evidence of lysis of organisms.

Secondly, in all the media chosen for this experiment as representative of the classes studied previously, morphology of the organism was remarkably homogeneous and of the species-type throughout the detectable loga-

rhythmic phase, with one exception, namely, in the complete medium (containing glucose and amino acids; medium no. 9) large numbers of bizarre forms were present even in young actively growing cultures.

Fig. 3. Distribution of cell-types of Fig. 1, in representative runs of mass population studies of *Ankistrodesmus falcatus* 188 (Indiana University Algal Culture Collection). Inocula were prepared by pooling and centrifugally washing 8-week cultures. Cultures were differentially counted after 8 weeks of growth.

In the other media, variant forms first appeared in the negative acceleration phase, and gradually increased in number throughout the later life of the culture. This was interpreted as indicating that substances which accumulated during growth caused the increasing variation in ageing cultures. An alternative hypothesis was that heretofore unreported sexual processes were causing genetic recombination and redistribution of genes in the population.

The characteristics of growth in this experiment were remarkably similar in the two media with N supplement (media no. 9 and 14); and different from those without (media no. 13 and 15). The curves illustrating this (Fig. 4) are separated by two log cycles for better visibility. In the unsupplemented media, growth stopped abruptly at approximately 10^7 organisms/ml. after about three to five major bursts of growth, each followed by lysis and quiescence of organisms before the next series of cleavages. In the N-supplemented media, however, growth never terminated;—rather, the number of organisms increased slowly during the entire course of the ‘stationary’ phase, giving final counts two to five times as high as in the non-supplemented media. In all media the degree of initial decrease in count, duration of log phase and rate of increase in logarithmic phase were approximately the same.

Table 3. *Stable and shifting predominance in populations of Ankistrodesmus falcatus on long-term incubation and successive subculture. Cell-types as percentage of total population*

Culture conditions	Cell-type no.	Strain and conditions of growth				
		101-A 70 days— medium 14	101-E 150 days— medium 14	101-G 70 days— medium 9	188-B 150 days— medium 14	188-F 70 days— medium 12
Original inoculum	1	96.8	96.8	96.8	0.69	0.69
	2	1.8	1.8	1.8	66.9	66.9
	9	—	—	0.0	—	—
21 days on GFS med. (control)	1	97.1	97.1	97.1	0.56	0.56
	2	1.8	1.8	1.8	75.84	75.84
	9	—	—	0.0	—	—
1—21 days on this med. (control)	1	96.9	96.9	68.7	4.76	1.67
	2	0.88	0.88	9.88	88.0	49.04
	9	—	—	1.77	—	—
2—150 days on this med.—duplicate of 1	1	—	70.75	—	0.0	—
	2	—	10.88	—	86.0	—
	9	—	—	—	—	—
3—70-day-old subculture from 1	1	0.52	—	0.0	—	87.14
	2	15.18*	—	80.0	—	10.0
	9	—	—	0.0	—	—
2 or 3 washed, resusp. in fresh min. med., inc. 21 days	1	1.0	75.81	0.0	0.0	91.0
	2	46.0	8.78	64.0	44.0	0.0
	9	—	—	1.0	—	—
21-day subcult. from 2 or 3 (this med.)	1	5.21	91.0	0.0	1.0	95.0
	2	44.79	0.5	78.0	58.0	1.0
	9	—	—	0.0	—	—
21-day trans. of 2 or 3 to min. med.	1	1.5	92.0	1.06	0.0	96.0
	2	62.0	4.0	88.23	70.0	1.0
	9	—	—	4.26	—	—
Comments		Type 1 up to 88.5 % in some transfers		Type 9 = 69.5 % on transfer of 3 to yeast; remained constant		

* Predominantly types 5 and 47.

Table 4. *Variation in individual morphology within clone of Ankistrodesmus braunii, 22 days' growth in mineral medium, first transfer after isolation*

Cell-type	Percentage of total population	Cell-type	Percentage of total population
2 ⁴⁶	0.2	28	0.2
3	90.8	39	1.0
7	1.0	50	0.4
10	0.4	58	4.4
18	0.2	54	0.8
19	0.2	55	0.2
24	0.2

Fig. 4

Fig. 4. Growth curves in tube cultures of *Ankistrodesmus braunii* 245-St 1.1, in various media at 400 f.c. continuous illumination and 24°. Curves are drawn for means of samples, plus and minus standard deviation. Counts during the first 10 days of growth were not obtainable because of mass lysis (see text). Inoculum was prepared by pooling and centrifugally washing 8-week cultures.

Fig. 5

Fig. 5. Growth curves in tube cultures of *Ankistrodesmus braunii* 245-St 1.1, at 400 f.c. continuous illumination and 24°, from inocula of varying size. The inoculum of 5×10^8 organisms/ml. was obtained by pooling and concentrating several cultures. All inocula were prepared by pooling and centrifugally washing 8-week cultures.

Normal development of *Ankistrodesmus braunii*

Anatomy of organisms. *Ankistrodesmus braunii* in the logarithmic phase of growth in mineral medium exhibits some features not clearly described previously. Typical individuals are shown in Pl. 1. They are fusiform, with a laminate, parietal chloroplast which extends nearly to each apex and about two-thirds around the periphery, much as in *Ulothrix*, etc. The chloroplast is capable of shifting position within the cell, so that it will come to lie in the bottom even when the individual is rolled over. At the equator of cell and chloroplast a prominent pyrenoid is located, usually surrounded by bead-like granules. Chains of granules ordinarily extend from the edge of the pyrenoid diagonally across the cell to the opposite wall, in the direction of either apex. Many conspicuous granules, crystals within vacuoles, etc., are usually present. The protoplast usually does not extend to the apices.

Division and growth (Pls. 1-3). In the early log-phase, the apex of the cell wall appears to soften and/or to excrete a slime, as the first indication of growth after introduction into fresh medium. This material is often of considerable volume and of a brownish colour, and may act as a holdfast when a suitable

substratum is present; but its secretion does not depend upon availability of attachment. Occasionally only one apex will develop a disk; and in spineless individuals (as in those arising from exsporulation) the apical material is often nearly or completely aborted. Elaboration of the apical material and growth of the organism are followed by the first cleavage, which begins at the pyrenoid and shortly after at a point directly opposite. The first cleavage furrow divides both protoplast and pyrenoid equally. It has not yet been possible to demonstrate a nucleus conclusively, in this or subsequent divisions.

The second cleavage furrows occur simultaneously, midway between the first furrow and either apex, within 1–2 hr. of the first furrow and parallel to it. The fate of the pyrenoid is difficult to trace in this and subsequent cleavages. Following second cleavage, the daughter cells ordinarily divide again with the next 2–3 hr. while continuing to grow; the average organism contains eight closely packed daughters, each having the shape impressed upon it by its neighbours and the confining mother cell-wall. The last set of furrows tends to be oblique to the transverse axis rather than parallel to it, so that many daughters are quite triangular at this time. Each of the progeny begins to elongate and grow in a common, apparently predetermined direction parallel to the long axis, resulting in a spiral configuration. Elongation corrects the cramped shape of the cells and causes the old cell-wall to split off as two empty, conical caps which may be retained by the growing cluster for some time. The newly liberated daughters grow for a period of 10–20 hr. before the first cleavage of the next division cycle. Division in progeny of a given mother tends to remain approximately synchronous for two or three division cycles. However, in any culture chamber some individuals produce a few daughters which may initiate the next cycle or continue the old one while elongating, spiralling and bursting the original wall.

Ageing and sporulation (Pl. 4). After four to six division-cycles have been completed, the daughter clusters or microcolonies tend to fall apart into discrete individuals. The secretion of apical material has steadily decreased with successive division cycles. At about the sixth cycle after inoculation, the chloroplasts become granular and oil droplets begin to appear in the cytoplasm. Both these phenomena obscure the pyrenoid of mature individuals. Organisms of this age were the only ones studied by many of the early workers; hence their description of this form as lacking a pyrenoid (Artari, 1892; Chodat, 1909, 1913). At this time many individuals do not elongate after division, but stay packed in the original cleavage configurations. Such individuals soon secrete heavy secondary walls and become aplanospores, as described by Klebs (1883) and Artari (1892). Still other vegetative cells form aplanospores without dividing, by rounding up the protoplast within the cell wall and forming a secondary internal wall. Such organisms have large empty spaces in the apices. As a sporulating culture ages, both spores and the remaining vegetative cells gradually lose the chlorophylls and all visible chloroplast structures and become filled with large yellow to orange fatty droplets. Many of the spores appear to retain the ability to divide vegetatively, liberating small spherical cells, thus explaining the report of Beijerinck (1890) con-

cerning the existence of a 'Chlorella cycle'. This cycle was particularly notable in the N-supplemented media, where the loss of green pigments was greatly retarded as well. Apparently in such cultures the mechanism of division is uncoupled from that of elongation.

Exsporulation (Pl. 5). Artari (1892) first observed the development of normal organisms from spores when he transferred dried spore clusters to fresh media in hanging-drop preparations. The pale, oil-packed organisms first undergo a rejuvenescence of pigments and chloroplasts essentially in the reverse order of events in the late stationary phase which led to bleaching and excessive fat deposition. Restoration of the smooth dark-green chloroplast may take from 2 to 5 days after inoculation into the culture chamber, and does not necessarily proceed at the same rate in all individuals of a spore cluster. Growth of the protoplast, after chloroplast function is restored, ruptures the spore case, in many instances before cleavage. The individuals liberated are approximately spherical when they originate from intercalary cells and conical to pisciform when originating from the apices. Frequently cleavage occurs while the germinating spore remains within the spore case. At any rate, elongation following cleavage produces tightly packed, coiled organisms, which soon burst the cell wall and retain their cramped form for only a short time; the normal events of growth and further elongation permit these cells to attain fairly normal shape, though many retain rather blunt apices. Progeny of the second division cycle are fully normal.

Abnormalities of spore germination and development are occasionally encountered, especially in some of the organic media. A few spores grow very large without cleavage and undergo a median constriction, which has never been seen to go to completion and give rise to two individuals. Other spores, after cleavage, liberate individuals which remain spherical, immediately lay down heavy spore walls, and repeat the process. The result is a cluster of cells in a *Protococcus*-like formation; only the original apical cells retain spines.

Abnormal vegetative cells. Most of the deviants from normal vegetative cells or stages associated with spores, fall into the category of triangular or multi-spinous cells. A small number of these is always present, especially from the third or fourth division cycle and later. Some of these may result from partial inhibition of cytokinesis, but many are apparently the product of the cramped configuration of daughters after cleavage. The cells located in the apices of the mother are most likely to be triangular or three-pronged in shape. Some of the progeny may retain this morphology, not because of genetic causes (though individual organisms might, of course, undergo a mutation) but usually because some of the plastic daughters are in turn pressed into the mould of the mother cell wall. The majority of progeny of a triangular individual is always of the species-type.

Lysis. Unpredictable and as yet uncontrollable numbers of organisms undergo spontaneous lysis even in control cultures (Pl. 6). This was the phenomenon which caused periodic decreases or plateaux in the net rate of increase in direct total count in the growth studies. At times cultures may be maintained for long periods in frequent serial transfer with little or no evidence

of lysis; at other times, this phenomenon appears like an epidemic. The majority of such organisms are seen in microculture to undergo apparently normal cleavage corresponding to the first division-cycle after inoculation, but without elongation and spiralling of daughters. Usually there are two or more additional cleavages, with a final yield of 64 or 128 daughters. In 48–72 hr. after inoculation, a weak point develops at the equator in the cell wall of the mother, and the daughters are liberated in a shower of protoplasmic clumps. Some individuals do not liberate formed clumps, but retain most of the cleavage products except for a slight ooze. These stages are reminiscent of abortive attempts at zoospore production in other algae.

Development in the presence of specific compounds

Effect of carbohydrates (Pls. 7 and 8). Only the standard concentration of glucose in the complex medium (0.04 %, w/v) permitted normal growth as compared with the controls, when the total numbers of organisms were counted in each chamber (Fig. 6). However, contrary to the earlier experiments, even this concentration gave significant numbers of variant shapes, of the types shown in Pl. 7, until the fourth day after inoculation. In the early runs, very young cultures in this medium were not examined because of great losses of organisms by lysis in all cultures. In microcultures the whole population appeared to adapt in the course of the first few division cycles, finally liberating only normally elongated daughters; the slight lag in growth as compared to the controls was corrected at this time.

Fig. 6

Fig. 6. The effect of varying concentrations of glucose upon growth of *Ankistrodesmus braunii* 245-St 1.1 in haemacytometer cultures. Counts were made of the total number of organisms in each chamber at intervals after inoculation. Inocula were prepared by diluting 1/50 the pooled sediment of three 72 hr. cultures.

Fig. 7

Fig. 7. The effect of varying concentrations of glucose upon morphology of *Ankistrodesmus braunii* 245-St 1.1 in haemacytometer cultures. Counts were made of the total number of normal organisms and deviants of any sort in each chamber at intervals after inoculation. Inocula were prepared by diluting 1/50 the pooled sediment of three 72 hr. cultures.

In the higher concentrations of glucose, the populations were more aberrant as the concentration increased (Fig. 7). Division was retarded at 0.4 % (w/v)

and produced daughters which appeared incapable of elongation. At 2.0 % (w/v) this effect was more pronounced, the resulting population consisting mostly of spherical, granular forms. A slight initial increase in numbers was followed by lysis of most organisms in what would have been the next division cycle. At 4.0 % (w/v) glucose appeared to inhibit altogether the ability to release daughters after cleavage. Many individuals lysed at an early stage, but many of those which survived went on to grow without further cleavage, producing a great bulge at the equator which continued to increase for as long as five or six weeks. All these stages were shown in Vischer's (1920) photographs. The quantitative series of effects of glucose may represent a serial uncoupling of the sequences related to cell division and release of normal daughters, similar to the sequences proposed for some of the yeast-like fungi by Nickerson (1948, 1953, 1954). They may be summarized diagrammatically as follows, where solid arrows show normal processes, and broken arrows show inhibition and results. Percentages shown are concentrations of glucose (w/v):

These effects were shown to be non-specific by growing organisms in the other hexoses and in penta-erythritol. Penta-erythritol in a range of concentrations equimolar with those of glucose used earlier gave precisely comparable effects, except that the time required was longer; for example, the 'great bulge' stage required about 4 weeks for initiation, as compared to 3–4 days in glucose. This difference may have been due to a difference in permeability to the two compounds. It was concluded from these experiments that the effect was an osmotic one, and comparable to osmotic effects upon protistan cell division noted by other workers (Whitfield & Murray, 1956; Schröder, 1954). In all cases, the possibility of selection of morphological mutants was ruled out repeatedly by transfer of organisms from experimental media to GFS medium; in microculture, all viable individuals produced daughters capable of elongation and other normal morphogenetic activities in the first or second division cycles.

Effect of various other compounds. Some non-amino acid components of the original organic test medium at different concentrations gave essentially the same effects in extremely low ($c. 10^{-7}$ – 10^{-8} M) concentration as did the highest concentration of glucose (2.2×10^{-2} M) (Table 5). Here, the effect could hardly have been osmotic, but might have been the result of blockage of specific metabolic pathways involved in morphogenesis. The effect appeared to be relatively non-specific, since the effective compounds were in the general category of C- and N-heterocyclic rings. The effective range of all the active compounds of this study is comparable to that of thymine antagonists for a thymineless strain of *Escherichia coli* (Cohen & Barner, 1956). This suggests that the nucleic acid-cobalamine-folic acid metabolic network may be one of

Table 5. *Effective levels of inhibition by heterocyclic compounds*

Substance	More than ($\mu\text{g./ml.}$)	Less than ($\mu\text{g./ml.}$)	Cytology
Control	—	—	Normal early stationary: fat deposition, sporulation
Azoles			
Imidazole	20	40	Normal cells but sparse
Pyridines			
Pyridine	10	20	Normal cells but sparse
Bipyridine	5	10	5 $\mu\text{g.}$: club-shaped, bulgy and normal cells; 10 $\mu\text{g.}$: no bulge—complete arrest of inoculum cells
Pyridoxine	40	?	Normal cells, slightly depressed growth
Purines			
Adenine	5	10	Small, swollen, bleached (arrested young 'monsters')
Guanine	?	1	Small, swollen, bleached (arrested young 'monsters')
Xanthine	1	2.5	Large, round 'monsters'
Pyrimidines			
Uracil	5	10	Bleached, lysing, arrested young 'monsters'
Thiamine HCl	10	20	Larger, round, lysed 'monsters'
Thymine	10*	?	Normal, early log-phase cells*

* KOH used to neutralize solution; may have resulted in hypertonicity at this concentration.

the systems involved. These and related compounds should be further screened to distinguish between quantitative parallel sensitivity of these sequences to the osmotic sequences, and the possibility that such compounds have an effect only upon metabolic pathways involved in some specific processes, such as cell elongation.

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R. McMILLAN—MORPHOGENESIS OF ANKISTRODESMUS. PLATE 1

(Facing p. 674)

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EXPLANATION OF PLATES

PLATE 1. Anatomy of *Ankistrodesmus braunii* from cultures in logarithmic phase, GFS medium. Magnification, $\times 900$.

- Fig. 1. Representative organisms after liberation from parent cell wall, showing old apical cap of mother, pyrenoids and typical chloroplast structure. Tube culture.
- Fig. 2. Organism in slide culture immediately before cleavage.
- Fig. 3. The same organism at same focal level five minutes later, showing shift in position of chloroplast.
- Fig. 4. Negative print from Kodachrome transparency of cell at time of inoculation into slide culture, showing lack of apical secretion.
- Fig. 5. Negative print from Kodachrome transparency of cell 15 min. after inoculation into slide culture, showing early apical secretion.

Fig. 6. Negative print from Kodachrome transparency of cell 2 hr. after inoculation into slide culture, showing secretion at both apices.

Fig. 7. Negative print from Kodachrome transparency of cell at second cleavage, showing growth and profuse apical secretion.

PLATE 2. Developmental history of a single average cell of *Ankistrodesmus braunii* in slide culture, GFS medium. Magnification $\times 500$, except fig. 13 which is $\times 220$.

Fig. 8. Regeneration of chloroplast and apical secretion in a cell from stationary phase culture, 32 hr. after inoculation.

Fig. 9. First cleavage, 48 hr.

Fig. 10. Products of first division cycle, 78 hr.

Fig. 11. Growth of cells in fig. 10, 104 hr.

Fig. 12. Second division cycle, 110 hr.

Fig. 13. Scattering and aberrant forms, 12 days.

PLATE 3. Cleavage and associated events in normal cells of *Ankistrodesmus braunii*. Magnification, $\times 600$.

Fig. 14. Synchrony of cleavage in sister cells, during second division cycle, with initiation of cleavage at pyrenoid. Tube culture.

Fig. 15. Configuration of cells in 4-cell stage. Tube culture.

Fig. 16. Configuration of cells in 8-cell stage. Slide culture.

Fig. 17. Spiralling of daughter cells. Slide culture.

Fig. 18. Three-pronged and triangular sister cells from fourth division cycle. Tube culture.

PLATE 4. Ageing and sporulation in *Ankistrodesmus braunii*. Magnification, various.

Fig. 19. Spiralling in a late division cycle with reduced elongation. 5 days, GFS medium. Magnification, $\times 350$. Slide culture.

Fig. 20. Degeneration of chloroplast and early formation of oil droplets. Progeny of the cell traced in Pl. 2, 13 days, GFS medium, slide culture. Magnification, $\times 350$.

Fig. 21. Division without elongation prior to sporulation, GFS medium, 7 days, slide culture. Magnification, $\times 350$.

Fig. 22. Mature spore clusters, ensporulated single cell and vegetative cell, packed with oil droplets. GFS medium, 130 days, tube culture. Magnification, $\times 700$.

Fig. 23. Spore cluster and spherical cells of 'Chlorella cycle' as described by Beijerinck; pigment and chloroplast structure normal. Yeast medium (medium no. 14), 130 days, tube culture. Magnification, $\times 700$.

Fig. 24. Representative oil-packed vegetative cells of stationary phase. GFS medium, 22 days, slide culture. Magnification, $\times 350$.

PLATE 5. *Ankistrodesmus braunii*: exsporulation. Magnification, various.

Fig. 25. Cleavage, spiralling, escape, elongation and initiation of next division cycle in a single germinating spore cluster. GFS medium, tube culture. Magnification, $\times 550$.

Fig. 26. Escape of protoplasts from spore-case prior to cleavage. GFS medium, slide culture. Magnification, $\times 550$.

Fig. 27. Cleavage of escaped protoplasts in a group similar to that of fig. 26. GFS medium, slide culture. Magnification, $\times 275$.

Fig. 28. A four-celled cluster with asynchronous development; growth and turgidity of intercalary cells have burst encasing the mother cell wall. GFS medium, slide culture, 6 hr. Magnification, $\times 275$.

Fig. 29. Escape of first group of progeny from cluster of fig. 28, 78 hr. Magnification, $\times 275$.

Fig. 30. Later division cycle and spiralling of first progeny; apical and right central cells pigmented. Magnification, $\times 275$.

Fig. 31. A pisciform cell representing apical cell of a spore cluster, just prior to first cleavage. GFS medium, slide culture, 49 hr. Magnification, $\times 275$.

Fig. 32. Progeny of cell in fig. 31, 78 hr. Magnification, $\times 275$.

PLATE 6. *Ankistrodesmus braunii*: abnormal spore development; abortive 'zoospores'. Magnification, various.

- Fig. 33. Large spore dividing (?) by constriction. Medium no. 14, tube culture, 130 days. Magnification, $\times 600$.
Fig. 34. Group of double-walled, spherical cells. Medium no. 9, slide culture, 70 days. Magnification, $\times 300$.
Fig. 35. *Protococcus*-like cluster. 4 % glucose (w/v) in GFS medium, slide culture, 28 days. Magnification, $\times 600$.
Fig. 36. Growth of spore protoplasts without release. GFS medium, tube culture, 96 hr.
Fig. 37. Three sister cells, one in cleavage, one releasing normal progeny, and one releasing 'zoospores'. GFS medium + $5\times$ normal concentration of $[\text{NO}_3]^-$, slide culture, 48 hr. Magnification, $\times 300$.

PLATE 7. Representative cell morphology of *Ankistrodesmus braunii* in varying concentrations of glucose. Magnification, $\times 750$, except fig. 41, which is $\times 375$.

- Fig. 38. Representative cells in 0.04 % glucose (w/v). Tube culture, 5 days. Cells in 0.4 % (w/v) were similar in morphology.
Fig. 39. Representative cells in 2.0 % glucose (w/v). Tube culture, 5 days.
Fig. 40. Representative cell in 4 % glucose (w/v). Tube culture, 28 days.
Fig. 41. Another representative cell in 4 %, showing extreme of protoplast extrusion and flaky degeneration of cell wall. Slide culture, 28 days.

PLATE 8. Effect of high osmotic pressure upon morphogenesis of *Ankistrodesmus braunii*: developmental history of variant cells. Magnification, $\times 700$.

- Fig. 42. Beginning of equatorial bulge; incipient first cleavage furrow. 4 % glucose (w/v), slide culture, 72 hr.
Fig. 43. Slightly older cell. 4 % glucose (w/v), slide culture, 96 hr.
Fig. 44. Representative cell, 4 % glucose (w/v), slide culture, 28 days. One intact, spherical daughter cell (which later divided), two naked portions of protoplasmic ooze and a large protoplasmic bulge have formed. Most of the chambers formed by early cleavages in region of original cell dimensions have been emptied.
Fig. 45. Another cell from the same slide culture as the cell in fig. 44; 28 days. Widely scattered lipid material and a small amount of protoplasmic ooze have been liberated. Many small, intact, spherical cells are retained within the mother cell.
Fig. 46. Beginning of equatorial bulge. Penta-erythritol, saturated solution, slide culture, 28 days. Comparable stages occurred in glucose, 4 % (w/v) at 48–72 hr.

PLATE 9. Protoplasmic ooze and zoospore formation: comparison of *Ankistrodesmus* and *Cladophora*. Magnification, $\times 900$.

- Fig. 47. Cluster of sister cells of *A. braunii* (in part, out of focus). Lateral equatorial bulges, with protoplasmic extrusion from one cell. 4 % glucose (w/v), slide culture, 28 days.
Fig. 48. Extrusion of protoplasmic ooze in *Cladophora* sp., in a filament of freshly collected material which was producing zoospores abundantly. The internal, flexible protoplasmic membrane has formed a 'sausage' within the cell; compare figs. 40, 44, and 47.
Fig. 49. Extruded protoplasm, a zoospore and a germling, from the same *Cladophora* filament shown in Fig. 48. The print was over-developed to render the flagella of the zoospore visible. Compare fig. 37.

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