Genet. Res., Camb. (1985), **46**, *pp.* 133–148
With 1 plate and 2 text-figures
Printed in Great Britain

Genetic investigation of a negatively phototactic strain of *Chlamydomonas reinhardtii*

BY ROBERT D. SMYTH

Department of Physics, Syracuse University, Syracuse, New York 13210

AND W. T. EBERSOLD

Department of Biology, University of California, Los Angeles, California 90024

(*Received 20 February 1985 and in revised form 3 June 1985*)

SUMMARY

Two laboratory strains of the green alga *Chlamydomonas reinhardtii 137c* differ in their pattern of phototactic aggregation. One is positively phototactic under conditions where the other is negatively phototactic. The trait segregates 2:2 in tetrads and maps to a single locus. Heterozygous diploids are positively phototactic, showing that this allele is dominant. The aggregation pattern caused by either allele is not altered by the introduction of an unlinked gene that suppresses development of the eyespot. Probably the strains already differed in phototactic behaviour at the time they were first isolated. They may therefore reflect a genetic polymorphism common among soil algae. The genetic data allowed another significant observation not specifically related to phototaxis. Anomalous products from some crosses suggest that four nuclei sometimes fuse into a tetraploid zygote that then undergoes meiosis. The meiotic products that result are diploid. This represents a previously undescribed mechanism of diploid formation in *Chlamydomonas*.

1. INTRODUCTION

Microscopic flagellated algae are phototactic: they can swim towards a source of light (positive phototaxis) or away from it (negative phototaxis). The response requires both a localized photoreceptor, which in most algae is sensitive to blue–green light, and adjacent structures that cause the light intensity on the photoreceptor to vary with the orientation of the cell to the light. Continuous rotation of the cell about an axis parallel to its swimming direction causes the light at the photoreceptor to rise and fall as the cell swims. This systematic modulation of the light detected by the photoreceptor provides the cell with the signal that it needs to orient its swimming path to the light direction. These aspects of phototaxis were discussed by Foster & Smyth (1980). Foster *et al.* (1984) have recently shown that a rhodopsin pigment is the receptor pigment for phototaxis in the green alga *Chlamydomonas*.

Chlamydomonas is a unicellular alga with two flagella. The flagella respond to a change in light intensity by transiently decreasing their beat frequency (Smyth

& Berg, 1982; Boscov & Feinleib, 1979; K. W. Foster, personal communication). Positively phototactic cells show an OFF response (slowed beating in response to a *decrease* in light intensity), whereas negatively phototactic cells show an ON response (slowed beating in response to an *increase* in light intensity). The two flagella are not identical (Huang *et al.* 1982; Kamiya & Witman, 1984). They are asymmetrically placed with respect to the photoreceptor, and one decreases its beat frequency more than the other in response to change in light intensity. This differential response of the two flagella causes the cell to turn. The position of the photoreceptor with respect to the flagella is critical for phototaxis. It must be so positioned that it achieves proper phasing between the light modulation cycle and the turning of the cell. The position of the photoreceptor ensures that when positively phototactic cells respond to a decrease in light intensity they turn towards the light. Negatively phototactic cells, on the other hand, respond in the opposite phase of the cycle when the light is increasing, and turn away from the light.

Beginning as far back as Strasburger (1876), observations on a variety of phototactic flagellates have shown that the sign of the phototactic response (positive or negative) is controlled by a two-position switch whose setting can change rapidly. (See Haupt, 1959, for review.) The change is clearly described in *Chlamydomonas* by Nultsch *et al.* (1971). The mechanism that controls the setting of this switch has been one of the most illusive problems of phototaxis. One promising approach is to isolate mutants with altered sign of phototactic response, on the assumption that physiological characterization of the mutants will provide clues to normal control. In this paper we report, as preliminary to such physiological study, a genetic analysis of two *Chlamydomonas* strains with opposite sign of phototactic response.

The strains were isolated in 1945 by G. M. Smith from a single colony of *Chlamydomonas reinhardtii* growing on agar. He labelled this colony *137c*. In *Chlamydomonas reinhardtii* sexual fusion occurs only between cells of opposite mating type, and mating type is determined by a single genetic locus (Smith & Regnery, 1950). Smith isolated two clones of opposite mating type, *WT*(+) and *WT*(−), from *137c*. The occurrence of cells of both mating types in this colony is presumptive evidence that it was formed by germination of a single zygote. These two strains and mutants derived from them constitute one of the three main groups, each of separate origin, of *Chlamydomonas reinhardtii* that are now used experimentally (Hoshaw, 1965; Sager, 1972). *WT*(+) and *WT*(−) from *137c* were maintained in vegetative culture without inbreeding. When we examined the behaviour of these two strains, we found that *WT*(−) was conspicuously more negatively phototactic than *WT*(+). The difference is caused by a single gene, *agg-1*, and is not linked to mating type.

A preliminary report of this work has appeared (Smyth & Ebersold, 1970). Hudock & Hudock (1973) and Hirschberg & Stavis (1977) have isolated mutant strains phenotypically similar to our negatively phototactic strain; these strains have not been mapped.

2. MATERIALS AND METHODS

(i) *Media and culture conditions*

Low-nitrogen minimal medium contained, per ml: 1·55 mg KH_2PO_4, 0·91 mg $K_2HPO_4 . 3H_2O$, 0·02 mg $MgSO_4 . 7H_2O$, 0·01 mg $CaCl_2 . 2H_2O$, 0·05 mg NH_4Cl, 0·001 ml trace-element solution (Hutner *et al.* 1950). Minimal medium contained in addition 0·45 mg/ml NH_4Cl. Yeast–acetate agar, routinely used to grow cultures, contained minimal medium plus 2 mg/ml Na acetate and 3 mg/ml Difco yeast extract. This and other solid media contained 15 mg/ml Difco agar. Zygotes for genetic analysis were matured on plates containing minimal medium plus 40 mg/ml agar. To score genetic markers by replica plating and spot testing, the following supplements were added as appropriate to minimal medium (per ml): nicotinamide 0·75 μg, L-arginine . HCl 10 μg, thiamine . HCl 1 μg, *p*-aminobenzoic acid 5 μg, DL-methionine-DL-sulphoximine 300 μg, streptomycin sulphate 50 μg, actidione 10 μg, L-canavanine . H_2SO_4 (Calbiochem) 1 mg, pyrithiamine . HBr (Calbiochem) 1 μg, oxythiamine . HCl 0·01 μg. Supplements were from Nutritional Biochemical Corporation, except as noted. Cultures were grown under Ken-Rad 30 W daylight fluorescent lamps at 6500 lux, 25–26 °.

(ii) *Aggregation test*

Segregants to be scored for *agg-1* were grown on yeast–acetate agar for about a week and transferred to fresh plates containing either yeast–acetate, for vegetative cells, or low-nitrogen agar, for gametes. On the following day, cells from the plates were suspended in 0·5 ml potassium phosphate buffer, 0·015 M, pH 6·7, at a concentration of $2–6 \times 10^6$ cells/ml. The suspensions were incubated at room temperature (20–21 °) in diffuse room light, provided by type CW fluorescent tubes in the ceiling at an illuminance of about 1600 lux. Cells showing the positive aggregation pattern (agg^+) formed a more or less uniform green suspension, whereas cells showing the negative aggregation pattern (agg^-) formed a compact green spot at the bottom of the tube with clear liquid above. The aggregation pattern usually became visible within 30 min, though the time was variable. Occasionally the pattern only became visible after 24 h.

(iii) *Tetrad analysis*

Techniques of mating, germination of zygotes, dissection of tetrads, and scoring of meiotic products are described by Ebersold & Levine (1959), Levine & Ebersold (1960), and Ebersold *et al.* (1962). Map loci and gene descriptions are given by Harris (1982). In crosses involving both *ac-17* and *ac-177*, *ac-177*$^-$ was identified by small colony size; *ac-17*$^+$, *ac-177*$^-$ grows slowly when spot-tested on minimal medium, whereas *ac-17*$^-$, *ac-177*$^-$ does not grow at all.

We use the abbreviations S for sensitive, R for resistant, PD for parental ditype, NPD for nonparental ditype, T for tetratype. Phenotypes are indicated by a gene symbol in parentheses.

Interpretation of the genetic data required prediction of the tetrad frequencies that would be produced by alternative genetic mechanisms. Such prediction requires that chiasma interference be taken into account. Of the many formulae that have been proposed to deal with chiasma interference, by far the best is that of Cobbs (1978). In this approach, the distribution of crossovers on a chromosome is approximated by a gamma distribution,

$$f(x) = \frac{\kappa^\kappa x^{\kappa-1}}{\Gamma(\kappa)} e^{-\kappa x} \tag{1}$$

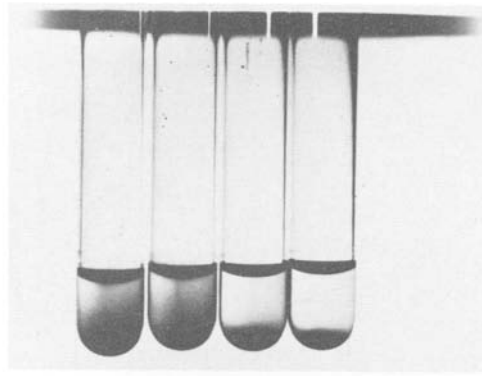
where x is genetic distance (mean number of crossovers) and $f(x)dx$ is the probability that the distance between two successive crossovers lies in the length range x to $x+dx$. For genetic purposes the number distribution is needed, the probability of n crossovers between two genetic markers, where $n = 0, 1, 2 \ldots$ This is calculated by assuming that crossovers are formed by a stationary renewal process. This means that the chromosome is modelled as a line segment of fixed length cut at random from an infinitely long line on which crossovers are distributed as described by the gamma distribution (see Cox & Lewis (1966) for the relevant theory). The parameter κ is a measure of chiasma interference and can range from $\kappa = 1$ for no interference (exponential distribution) to $\kappa = \infty$ for constant distance between crossovers. Cobbs showed that the gamma distribution gives excellent fit to data for *Neurospora* ($\kappa = 3$) and *Drosophila* ($\kappa = 6$). We find that the gamma distribution with $\kappa = 15$ gives excellent fit to the data of Ebersold & Levine (1959) on four linked genes in *Chlamydomonas*, and we use this to estimate expected frequencies. When $\kappa = 15$, the maximum ratio of NPD to T for linked markers is 0·32. We therefore used NPD/T $\geqslant$ 0·32 as a criterion for the absence of linkage. (We do not report expected frequencies of products from triploid or tetraploid meioses because non-viable products in the crosses where such meioses occurred complicated the analysis.)

(iv) *Monte Carlo chi-square statistics*

We used the chi-square method to choose between different interpretations of genetic data. Usually, however, the data included categories so small that the χ^2 probability could not be reliably estimated from the theoretical χ^2 distribution. In these cases we estimated the probability by Monte Carlo simulation. In effect we repeated the experiment many times by computer simulation, based on the statistical assumptions of the hypothesis being tested, and observed whether results that deviated from expectation by as much as the experimental data were rare or common. Specifically, if there are N events (tetrads or single products) and K observable kinds of event (categories), then

$$\chi^2 = N \sum_{i=1}^{K} (p_i - n_i/N)^2/p_i \tag{2}$$

where p_i is the hypothetical probability that an event is in category i, and n_i is the observed number of events in category i. Using a PDP-11/34 computer, we simulated 10000 independent draws of N events from a population of events with



Segregation of *agg-1* in a single tetrad. The two meiotic products on the left are *agg-1*$^{+}$; the two on the right are *agg-1*$^{-}$. (See Materials and Methods, *Aggregation test.*)

https://doi.org/10.1017/S001667230002262X Published online by Cambridge University Press

probabilities p_i. For each set of N events we calculated χ^2 and estimated the χ^2 probability as the proportion of the sets in which the simulated χ^2 was equal to or greater than the observed χ^2.

2. RESULTS

(i) *Behaviour*

When cells of the negatively phototactic strain were suspended in a small volume of buffer, as described above, the cells aggregated in a small spot in the bottom of the tube with clear supernatant above (Plate 1, two tubes on right). The aggregation required light. This requirement was most clearly demonstrated with suspensions that had stood in the light for 24 h. When a tube containing aggregated cells was shaken to resuspend the cells, the cells re-aggregated in less than 5 min. If, after shaking, the tube was immediately placed in a light-tight box, no re-aggregation occurred in half an hour, but the cells aggregated in less that 5 min when returned to the light. When a suspension was illuminated from below, the green spot did not form; cells aggregated to some extent at the surface, but convective streaming from the dense surface layer caused so much mixing that the entire suspension remained green. When a cell suspension in a petri dish or under a coverslip was illuminated from the side, the cells formed a tight aggregation away from the light.

The aggregation pattern of the positively phototactic strain was more variable. When suspended in buffer, the cells remained in more or less uniform suspension, with some surface aggregation and convective streaming (Plate 1, two tubes on left). When illuminated from below, the cells remained in suspension and did not form a spot in the bottom of the tube. In a petri dish or on a slide, suspensions illuminated from the side usually aggregated only on the side towards the light, but sometimes formed visible aggregations on both the side towards the light and the side away from it. When the light direction was reversed in such preparations, the cells in both aggregations swam to opposite sides.

(ii) *Segregation*

In all crosses between positively and negatively phototactic strains, the behavioural difference segregated 2:2 (Plate 1), indicating control by a single genetic locus. We examined about 400 tetrads in which all four meiotic products were scored. We named the allele causing negative phototactic aggregation *agg-1*$^-$ and the opposite allele *agg-1*$^+$.

There was no obvious morphological difference between *agg-1*$^+$ and *agg-1*$^-$ cells. The colonies were identical in size and colour. The cells appeared identical when examined in the light microscope, and the position of the eyespot was the same in both.

(iii) *Linkage*

Agg-1 was crossed with strains containing markers in 17 linkage groups. The results are summarized in Table 1. Inspection of the observed PD:NPD:T

frequencies shows that *agg-1* is in linkage group XIV (PD ≫ NPD) (Perkins, 1953). The probabilities in columns 4 and 5, calculated from the binomial distribution, give quantitative information about linkage and non-linkage. The probabilities in column 4 are tests of the hypothesis that *agg-1* and the test marker *are not* linked

Table 1. *Segregation of* agg-1 *in crosses with markers in 17 linkage groups*

Linkage group	Marker	PD:NPD:T	Probability (PD = NPD)*	Probability (NPD/T = 0·32)†
I	*ac-14c*	12:10:9	0·83	0·007
II	*pf-12*	9:15:13	0·31	0·0008
III	*ac-17*	44:57:2	0·23	8×10^{-32}
IV	*pyr-1*	49:39:15	0·34	$1·5 \times 10^{-13}$
V	*pf-1*	6:4:14	0·75	—
VI	*mt*	20:12:28	0·22	0·25
VII	*pf-17*	9:9:3	1·00	0·0003
VIII	*pf-3*	10:5:11	0·30	0·34
IX	*str-1a*	17:19:16	0·87	0·0001
X	*nic-13*	12:11:4	1·00	0·0001
XI	*pf-2*	8:9:4	1·00	0·0008
XII	*ac-9*	10:5:9	0·30	0·24
XIII	*nic-15*	4:3:4	1·00	0·23
XIV	*ac-177*	73:0:30	10^{-22}	—
XV	*nic-1*	11:14:26	0·69	0·084
XVI	*ac-46*	9:10:1	1·00	6×10^{-6}
XVII	*y-1*	57:44:2	0·23	5×10^{-25}

*Probability PD = NPD is the probability of obtaining a PD:NPD ratio that deviates from 1:1 by as much as or more than the observed ratio, on the assumption that PDs and NPDs are equally probable.

†Probability NPD/T = 0·32 is the probability of obtaining NPD/T as great as or greater than the observed value if the expected value of NPD/T is 0·32 (the maximum value for linked genes if $\kappa = 15$). NPD/T < 0·32 is consistent with either linkage or non-linkage.

(PD and NPD equally probable). The probabilities for all groups except XIV are consistent with non-linkage. The probabilities in column 5 are tests of the hypothesis that *agg-1* and the test marker *are* linked. For linked genes, NPD/T can range from 0 to 0·32 (if $\kappa = 15$); for unlinked genes, NPD/T has no upper limit. A value significantly greater than 0·32 is therefore evidence of non-linkage. The low probabilities associated with 10 linkage groups in column 5 provide evidence of non-linkage. In the other seven groups the NPD/T test is consistent with either linkage or non-linkage.

Two markers besides *agg-1* are reported for linkage group XIV, *ac-177* and *ac-206* (Harris, 1982). We were unable to recover acetate-requiring products from crosses with *ac-206*. Therefore we determined the order and map distances of *agg-1*, *ac-177* and the centromere, the only mapping possible in linkage group XIV. To score centromeres in tetrads one must identify the two pairs of meiotic products that separate at the first meiotic division, during which the daughter chromatids from each parent remain attached at the centromere. We identified these pairs by using centromere markers, genes so close to the centromere that they usually segregate at the first division. We used *ac-17* in linkage group III and *y-1* in linkage group

XVII (Ebersold *et al.* 1962; Sager, 1955). The use of centromere markers complicates the analysis because one cannot assume that crossovers between the centromere and the marker never occur, even though they are rare.

We made the folllowing two crosses:

$$agg\text{-}1^{+},\ ac\text{-}177^{-},\ ac\text{-}17^{+},\ y\text{-}1^{+},\ mt^{+} \times agg\text{-}1^{-},\ ac\text{-}177^{+},\ ac\text{-}17^{-},\ y\text{-}1^{-},\ mt^{-} \quad (1)$$

$$agg\text{-}1^{-},\ ac\text{-}177^{+},\ ac\text{-}17^{-},\ y\text{-}1^{-},\ mt^{+} \times agg\text{-}1^{+},\ ac\text{-}177^{-},\ ac\text{-}17^{+},\ y\text{-}1^{+},\ mt^{-} \quad (2)$$

We plated 80 zygotes from each cross and scored 103 tetrads. The phenotypes of these 103 tetrads are shown in Table 2. (*Ac-17* and *y-1* are listed only as ditype or tetratype, since the PD:NPD ratio for unlinked genes (theoretically 1:1) contains no relevant information). High chiasma interference in *Chlamydomonas* simplifies the analysis: it is extremely unlikely that more than one crossover per chromosome per tetrad occurred in any of the intervals considered. For example, the longest possible interval is between *ac-177* and the centromere, with a map distance of $50 \times (31/103) = 15$ centimorgans. The theoretical probability of a double crossover in this interval is of the order of 10^{-6}. In Table 3 we list three alternative hypotheses about the order of the genes and show the corresponding probabilities of obtaining the observed tetrad set. Because the χ^2 probability of hypothesis A is 0·52 and the χ^2 probabilities of hypotheses B and C are both less than 0·05, we accept hypothesis A and conclude that the order is as shown in Fig. 1.

Table 2. *Tetrad categories observed in crosses (1) and (2)*

Tetrad category	Marker pair: *agg-1* *ac-177*	*agg-1* *ac-17*	*agg-1* *y-1*	*ac-177* *ac-17*	*ac-177* *y-1*	*ac-17* *y-1*	Number observed
1	PD	D	D	D	D	D	71
2	T	D	D	T	T	D	29
3	PD	T	D	T	D	T	1
4	PD	T	T	T	T	D	1
5	T	D	T	T	D	T	1
							103

D = Ditype (PD + NPD)

(iv) *Dominance*

Chlamydomonas is haploid during vegetative growth. It is diploid only in the zygote, a single cell formed after the fusion of two haploid gametes. The zygote is motile for a time and then forms a thick-walled resting spore, which upon germination immediately undergoes meiosis. Since the normal life cycle contains no propagating diploid stage, special techniques are required to test dominance. We constructed diploids by mating two strains with different nutritional requirements, plating on minimal agar, and picking prototrophic colonies (Ebersold, 1967). This procedure selects from the mating mixture rare cells in which fusion of two cells and their nuclei was followed by vegetative growth rather than by

meiosis. For reasons that will become clear, we call such cells *ameiotic diploids*. Besides diploids, haploids that have become prototrophic as a result of meiotic recombination can also grow on minimal plates. To avoid these, we used parental strains that were heterozygous for 10 genes besides *agg-1* (Fig. 2). These were chosen

Table 3. *Location of the crossovers that produced the tetrads in Table 2 according to three alternative hypotheses*

(Hypothesis A, *agg-1* and *ac-177* are in the same chromosome arm; Hypothesis B, *agg-1* and *ac-177* are in opposite arms; Hypothesis C, no crossover occurred between *agg-1* and the centromere, so the order of *agg-1* and the centromere cannot be determined from the data.)

		Interval						
Hypothesis	Tetrad category	*agg-1* cent.	*ac-177* *x*	*ac-17* cent.	*y-1* cent.	*n*	χ^2	*P*
A	1	0	0	0	0	71	5·2	0·52
	2	0	1	0	0	29		
	3	0	0	1	0	1		
	4	1	0	0	0	1		
	5	0	1	0	1	1		
Crossover frequency		1/103	30/103	1/103	1/103			
B	1	0	0	0	0	71	72	0·034
	2	0	1	0	0	29		
	3	0	0	1	0	1		
	4	0	0	1	1	1		
	5	1	0	1	0	1		
Crossover frequency		1/103	29/103	3/103	1/103			
C	1	0	0	0	0	71	100	0·029
	2	0	1	0	0	29		
	3	0	0	1	0	1		
	4	0	0	1	1	1		
	5	0	1	0	1	1		
Crossover frequency		0	30/103	2/103	1/103			

x = *agg-1* in hypothesis A, centromere in hypothesis B, *agg-1* or the centromere in hypothesis C.

If each chromosome has at most one crossover in the marked interval and the centromere markers are scored only as ditype or tetratype, there are 14 observable kinds of tetrad ($K = 14$ in Formula 2). The 5 categories actually observed are listed (n = no. observed). The number of crossovers (0 or 1) in each interval is shown for each category. For each hypothesis, we calculated the probability of occurrence of each of the 14 categories (not shown) on the basis of the crossover frequencies listed and independent assortment of the unlinked genes. From these probabilities we could calculate χ^2 for any possible set of 103 tetrads ($N = 103$ in Formula 2). P is the proportion of 10000 sets of 103 tetrads drawn by Monte Carlo simulation that had χ^2 greater than χ^2 of the observed set of tetrads (which is listed).

(*a*) to reduce the number of prototrophic haploid recombinants and (*b*) to give to prototrophic haploid recombinants phenotypes different from diploids.

Four strains were constructed: two mt^+ strains, identical except that one was *agg-1*$^+$, the other *agg-1*$^-$; and two mt^- strains, likewise different only in *agg-1*$^+$ and *agg-1*$^-$. The mt^- strains required arginine, *p*-aminobenzoic acid, thiamine, and nicotinamide, and were yellow because they contained *ac-29a*. The mt^+ strains

required acetate and were resistant to methionine sulphoximine, actidione and streptomycin. The five markers in linkage group I were in staggered configuration (Fig. 2). A prototrophic recombinant could be formed only by a double crossover in the intervals *arg-1-ac-14* and *ac-14-pab-2*, an event with theoretical expectation of only 4×10^{-4}. Such a recombinant would be methionine sulphoximine-resistant

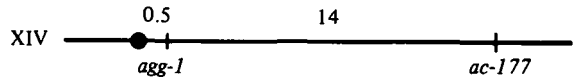


Fig. 1. Map location of *agg-1* in linkage group XIV. Numbers indicate map distances in centimorgans based on Table 3, hypothesis A. The centromere is indicated by a solid circle.

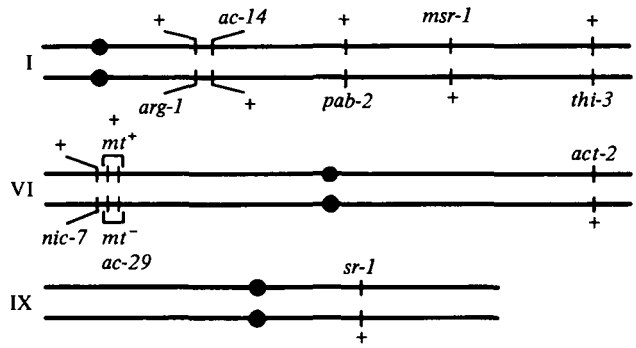


Fig. 2. Location of the genetic markers used to construct diploids. Linkage groups are indicated by roman numerals. The centromeres are indicated by solid circles. In each group the upper line represents markers from the mt^+ parent, the lower line from the mt^- parent. The order of *mt* and *ac-29* is not known. Lengths in diagram are approximately proportional to map distances.

because four crossovers would be required to produce a methionine sulphoximine-sensitive prototroph, an event of vanishingly small probability. Prototrophic haploid recombinants could be either resistant or sensitive to actidione and streptomycin. The drug-resistance markers are recessive at the concentrations used (Martinek, Ebersold & Nakamura, 1970; Lee *et al.* 1973; see below). Ameiotic diploids should therefore be sensitive to methionine sulphoximine, and also to actidione and streptomycin. Ameiotic diploids are (mt^-) (Ebersold, 1967). Most prototrophic haploid recombinants should be (mt^+), because formation of (mt^-) recombinants would require a crossover between *nic-7* and *mt*, with probability of only about 2×10^{-3} (Smyth, Martinek & Ebersold, 1975).

In summary, the parental strains were designed so that ameiotic diploids would have the phenotype (mt^-, msr^S, act^S, str^S), whereas prototrophic haploid recombinants would be (mt^+, msr^R, $act^{S\text{ or }R}$, $str^{S\text{ or }R}$). We expected that most prototrophic colonies would be ameiotic diploids and that only a few, or more likely none at all, would be haploid recombinants. This was partly borne out, in that 186 out of 214 prototrophic colonies had phenotypes expected of ameiotic diploids and five had phenotypes expected of haploid recombinants (Table 4). A completely unexpected result was that 23 colonies did not fall into either category. Apparently a third process produced prototrophic colonies, at a frequency lower than ameiotic

diploids and at a much higher frequency than haploid recombinants. The classification shown in Table 4 of the 214 prototrophs would appear to be the simplest interpretation of the data. We classify 184 as ameiotic diploids because they have the expected phenotype and show a consistent pattern of dominance

Table 4. *Phenotypes of presumptive diploids picked from minimal agar plates*

	Strains crossed	Unselected marker					No. of colonies
		msr	*str*	*act*	*mt*	*agg*	
Ameiotic diploids	(1) × (3)	S	S	S	−	+	56
	(1) × (4)	S	S	S	−	+	51
	(2) × (3)	S	S	S	−	+	35
	(2) × (4)	S	S	S	−	−	42
							184
Meiotic diploids	(1) × (3)	R	S	S	+	+	2*
		S	R	S	−	+	1
	(2) × (3)	S	S	S	+	+	4
		R	S	S	+	+	2*
		R	R	S	+	+	1*
		S	S	R	+	+	1
		R	S	S	−	+	1
		S	R	S	−	+	4
		S	S	R	−	+	8
		R	S	R	−	+	2
		S	S	R	+	−	1
		S	S	S	−	−	2†
		S	R	S	−	−	1
							30

*Met^R, mt^+ strains could be haploid meiotic recombinants.
†Met^S, str^S, act^S, mt^- is also consistent with ameiotic diploid origin.
Parental strains:
(1) *ac-14c, msr, act-2, str-1a, agg-1*$^+$, *mt*$^+$
(2) *ac-14c, msr, act-2, str-1a, agg-1*$^-$, *mt*$^+$
(3) *arg-1, pab-2, thi-3, nic-7, ac-29a, agg-1*$^+$, *mt*$^-$
(4) *arg-1, pab-2, thi-3, nic-7, ac-29a, agg-1*$^-$, *mt*$^-$
The parental strains were transferred to low-nitrogen agar supplemented with arginine, Na acetate, *p*-aminobenzoic acid, thiamine and nicotinamide, and incubated overnight. The cells were then suspended in 1 ml sterile water, concentration about 4×10^6 cells/ml. After 1 h under fluorescent lights, the parental strains were mixed and stirred frequently. Aliquots were withdrawn at half hour intervals for 3 h; one drop of suspension and one drop of a 1:10 dilution of the suspension were spread on minimal agar plates containing oxythiamine and pyrithiamine to suppress growth of *thi-3*. When colonies appeared, about 60 colonies from each cross were picked, spread on minimal agar containing oxythiamine and pyrithiamine, and single cells isolated with a glass loop.

of *agg-1*$^+$. The other two prototrophs that could be ameiotic diploids and the five possible haploid recombinants occur at about the same frequency as the 23 anomalous prototrophs and could all be formed by the same process. We therefore lump all 30 of these strains together under 'meiotic diploids' and discuss them in section (vi).

All apparent ameiotic diploids gave unambiguous results in the aggregation test. Homozygous *agg-1*$^+$ diploids were (agg$^+$) and homozygous *agg-1*$^-$ diploids were

(agg$^-$). The diploid condition therefore does not interfere with expression of the trait. The 86 ameiotic diploids from parents heterozygous for *agg-1*$^+$ and *agg-1*$^-$ were all phenotypically (agg$^+$), showing that *agg-1*$^+$ is dominant over *agg-1*$^-$.

Three lines of evidence confirm the diploid character of the 184 ameiotic diploids: low viability of products formed in crosses to haploid wild type, appearance of hidden recessives among these products, and appearance of drug-resistant segregants during vegetative growth. We next consider each of these in turn.

We crossed four of the apparent ameiotic diploids to haploids. In diploid by haploid crosses, the zygote nucleus contains six copies of each chromosome. The daughter cells receive at random either one or two copies (occasionally zero or three) (Fincham & Day, 1965). The resulting genetic imbalance produces low viability and abnormal colonies (Ebersold, 1967). We crossed one ameiotic diploid from each parental combination to haploid *agg-1*$^+$ wild type, and isolated a total of 1000 zygotes on yeast–acetate agar plates. Almost all zygotes germinated, but less than half formed visible colonies. The colonies that did form varied greatly in size, texture and colour, many showing slow growth. This behaviour is typical of zygotes from diploid by haploid crosses and unlike that of zygotes from haploid by haploid crosses, which have higher viability and form colonies of more uniform appearance.

We looked for hidden recessives by making single-cell isolates from 158 of the zygote colonies and testing for all markers (Table 5). The recessive phenotypes (ac$^-$), (nic$^-$), (thi$^-$), (msr^R), (act^R), (str^R) were found among the products. No (arg$^-$) or (pab$^-$) products were found: the parental *arg-1*$^-$ strains grew very slowly: slow growth of *arg-1* probably prevented recovery of either *arg-1*$^-$ or its linked marker *pab-2*$^-$. We could not score *ac-29a* because of variability in the colour of the colonies. The genetic imbalance of these strains also caused variability in phototactic behaviour, making them difficult to score in the aggregation test. Nevertheless, as shown in Table 5, some products from the *agg-1*$^+$/*agg-1*$^-$ × *agg-1*$^+$ and *agg-1*$^-$/*agg-1*$^+$ × *agg-1*$^+$ crosses appeared to be (agg$^-$) (11 out of 97). Of 17 products from the *agg-1*$^-$/*agg-1*$^-$ × *agg-1*$^+$ cross, 7 were (agg$^-$). None of the 44 products from the *agg-1*$^+$/*agg-1*$^+$ × *agg-1*$^+$ cross was (agg$^-$). The results of the diploid by haploid crosses are therefore consistent with dominance of *agg-1*$^+$.

When spot-tested on agar plates containing methionine sulphoximine, actidione or streptomycin, the putative ameiotic diploids initially showed no visible growth, but later the spot acquired a speckled appearance. The speckled spot was clearly different from either the solid green growth of resistant haploids, or the lack of growth of sensitive haploids. Cells scraped from the speckled spots and retested were resistant. The speckled appearance is caused by secondary growth of resistant colonies, presumably as a result of mitotic nondisjunction (Martinek, Ebersold & Nakamura, 1970; Lee, Whiteway & Yorke, 1976; Matagne & Orbans, 1980). Because the speckled trait was somewhat variable, we could not score all products. Many of the aneuploid products of diploid by haploid crosses also formed speckled spots. (Eves & Chiang (1982) assumed that the resistant alleles of *msr* and *str* are dominant or codominant, and obtained segregation frequencies consistent with this assumption. They may have scored heterozygotes as resistant because of the

Table 5. *Products recovered from diploid by haploid crosses*

Marker								Number of products: Cross				
ac	*msr*	*thi*	*str*	*act*	*nic*	*mt*	*agg*	A	B	C	D	Total
+	S	+	S	S	+	+	+	5	6	12	2	25
−	S	+	S	S	+	+	+	1	·	4	·	5
+	R	+	S	S	+	+	+	2	3	5	1	11
−	R	+	S	S	+	+	+	10	3	9	2	24
+	S	−	S	S	+	+	+	·	1	·	·	1
+	S	+	R	S	+	+	+	1	2	4	·	7
−	S	+	R	S	+	+	+	·	·	1	·	1
+	R	+	R	S	+	+	+	1	·	·	1	2
−	R	+	R	S	+	+	+	2	·	3	·	5
−	R	−	R	S	+	+	+	·	·	1	·	1
+	S	+	S	R	+	+	+	·	·	1	1	2
−	S	+	S	R	+	+	+	·	1	·	·	1
+	R	+	S	R	+	+	+	·	1	·	·	1
−	R	+	S	R	+	+	+	·	·	2	1	3
+	S	−	S	R	+	+	+	·	·	1	·	1
+	S	+	R	R	+	+	+	·	1	·	·	1
+	R	+	R	R	+	+	+	·	1	1	1	3
−	R	+	R	R	+	+	+	·	·	2	·	2
−	S	−	R	R	+	+	+	·	·	1	·	1
−	R	−	R	R	+	+	+	·	·	1	·	1
+	S	+	S	S	+	−	+	5	4	4	1	14
−	S	+	S	S	+	−	+	2	·	·	·	2
+	R	+	S	S	+	−	+	1	1	2	·	4
−	R	+	S	S	+	−	+	·	·	1	·	1
+	R	−	S	S	+	−	+	·	1	·	·	1
+	S	+	R	S	+	−	+	2	·	2	·	4
+	R	+	R	S	+	−	+	1	·	1	·	2
−	R	+	R	S	+	−	+	1	·	·	·	1
+	S	+	S	R	+	−	+	·	·	1	·	1
+	S	+	S	S	−	−	+	1	·	2	·	3
−	R	+	S	S	−	−	+	6	·	·	·	6
−	S	−	S	S	−	−	+	1	·	·	·	1
−	R	−	S	S	−	−	+	1	·	·	·	1
−	R	+	R	S	−	−	+	1	·	·	·	1
+	S	+	S	S	+	+	−	·	2	·	1	3
+	R	+	S	S	+	+	−	·	·	·	1	1
−	R	+	S	S	+	+	−	·	·	·	1	1
−	S	−	S	S	+	+	−	·	·	1	·	1
+	S	+	R	S	+	+	−	·	1	2	·	3
+	R	+	R	S	+	+	−	·	·	·	2	2
−	R	+	R	S	+	+	−	·	1	·	·	1
−	S	+	S	R	+	+	−	·	·	·	1	1
−	R	+	S	R	+	+	−	·	·	·	1	1
+	S	+	R	R	+	+	−	·	1	·	·	1
−	R	+	R	R	+	+	−	·	1	·	·	1
+	R	+	S	S	+	−	−	·	1	·	·	1
−	R	+	S	R	+	−	−	·	·	1	·	1
								44	32	65	17	158

Genetic constitution of strains used in diploid by haploid crosses: all diploids were *ac-14c,msr,act-2, str-1a/arg-1,pab-2,thi-3,nic-7,ac-29a*; in addition they contained *agg-1* and *mt* in the following combinations: cross A, *agg-1*$^+$,*mt*$^+$/*agg-1*$^+$,*mt*$^-$ × *agg-1*$^+$,*mt*$^+$; cross B, *agg-1*$^+$,*mt*$^+$/*agg-1*$^-$,*mt*$^-$ × *agg-1*$^+$,*mt*$^+$; cross C, *agg-1*$^-$,*mt*$^+$/*agg-1*$^+$,*mt*$^-$ × *agg-1*$^+$,*mt*$^+$; cross D, *agg-1*$^-$,*mt*$^+$/*agg-1*$^-$,*mt*$^-$ × *agg-1*$^+$,*mt*$^+$. Individual zygotes from each cross were separated on yeast-acetate agar by drawing the zygotes into lanes with a fine glass loop, 100 zygotes per plate. Meiotic products were not separated, but formed a single mixed colony. Cross A gave 123 zygote colonies from 300 zygotes; cross B, 164 colonies from 300 zygotes; cross C, 82 colonies from 200 zygotes, cross D, 37 colonies from 200 zygotes. Clones derived from a single cell from 158 different zygote colonies were scored. All products were (arg$^+$) and (pab$^+$). The diploid in cross A had apparently lost the *act-2* gene.

https://doi.org/10.1017/S001667230002262X Published online by Cambridge University Press

secondary formation of resistant cells, i.e. they may have scored our 'speckled' as 'resistant'.)

(v) *Combination of* agg-1 *with* ey

Chlamydomonas has a pigmented eyespot on the side of the cell. The eyespot enhances optical contrast at the photoreceptor by absorption of light and by interference reflection from the layered surfaces of the eyespot (see Foster & Smyth, 1980). Hartshorne (1953) isolated a mutant, *ey*, with a defective eyespot that showed impaired phototaxis. *Ey* is in linkage group X, not linked to *agg-1* (Smyth, Martinek & Ebersold, 1975). After combining ey^- and $agg\text{-}1^-$, we crossed $agg\text{-}1^+$, ey^+, mt^+ with $agg\text{-}1^-$, ey^-, mt^- and scored 37 tetrads. Both *ey* and *agg-1* showed normal 2:2 segregation in all tetrads. No new or intermediate phenotype appeared in the aggregation test. *Ey* apparently has no noticeable effect on the behaviour of $agg\text{-}1^+$ and $agg\text{-}1^-$ strains in this test. This result is not inconsistent with the observation of impaired phototaxis in *ey* strains by Hartshorne (1953) and Morel-Laurens & Feinleib (1983). These workers measured the rate of phototactic aggregation, whereas we observed only the final state. Although the eyespot is an important source of contrast at the photoreceptor, it is not the only source. Absorption of light by chloroplast pigments, scattering and refraction by the cell body, and possibly orientation of the photoreceptor pigment molecules also produce contrast. Phototaxis is therefore possible without the eyespot, but less efficient. Our observations suggest that some other component of the phototaxis apparatus than the eyespot is different in $agg\text{-}1^+$ and $agg\text{-}1^-$.

(vi) *Meiotic diploids*

Thirty colonies out of the 214 obtained by the procedure designed to isolate ameiotic diploids (see (iv) *Dominance*) did not appear to be ameiotic diploids. Only five, at most, could be haploid recombinants. These 30 colonies cannot be explained on any simple pattern of back mutation, gene conversion or mitotic recombination, and seem to represent a new category of product. We believe they are *meiotic diploids*, diploid products of meiosis. They did not have the phenotype of ameiotic diploids, but resembled ameiotic diploids in having a growth habit indistinguishable from wild type and in giving unambiguous results in the aggregation test. This suggests that they had a euploid chromosome number. Many of them formed speckled spots when spot-tested on methionine sulphoximine, actidione or streptomycin plates, indicating the presence of alleles for both drug sensitivity and resistance. The strains were discarded before they could be tested further. We believe these strains were formed at meiosis in the following way. *Chlamydomonas* cultures always contain a certain number of twinned cells, cells with a pair of flagella at either end, and the number of twinned cells varies widely from culture to culture (Lewin, 1952; Sager, 1955; Schaechter & DeLamater, 1956; Warr, 1968). Warr showed that twinned cells are binucleate, and that each end can mate. (See also Gillham (1978), fig. 12-6.) Fusion of one twinned cell with two uninucleate cells could produce a tetraploid nucleus, which would give rise to diploid products by meiosis.

The same process of tetraploid meiosis could explain an observation made by Ebersold (unpublished) of zygotes that produce complete tetrads containing markers from three different haploid strains present in the same mating mixture.

4. DISCUSSION

We have shown that two strains of *Chlamydomonas reinhardtii 137c* have a conspicuously different pattern of phototactic aggregation, and that the difference is controlled by a single gene, *agg-1*. Probably the strains already showed this difference at the time they were isolated in 1945, although this cannot be proved now. One reason this seems likely is that the two alleles have been completely stable during vegetative culture since the behavioural difference was first observed 19 years ago. Moreover, these alleles are found scattered among the mutant strains that were derived early from *137c*. Most of these strains are positively phototactic (agg^+), but some are negatively phototactic (agg^-). Whether both positively and negatively phototactic strains are found in nature is not known. Testing the phototactic aggregation of a large number of clones of *Chlamydomonas* derived from fresh isolates would probably confirm that both types are present.

The methods used to study *agg-1* illustrate some of the advantages of a sexual organism like *Chlamydomonas* for investigating photobehaviour, and suggest some new approaches for future studies. A question basic to understanding any kind of behaviour is the extent to which the behaviour is genetically or environmentally determined. This is especially important in phototaxis, which is sensitive to environmental conditions. The genetic basis of the difference in our positively and negatively phototactic strains was easy to demonstrate because *Chlamydomonas* could be crossed and techniques were available for isolating meiotic products. A powerful aid to this demonstration was the ability in *Chlamydomonas* to analyse individual tetrads, because genetic traits segregate in exact ratios in tetrads. Final proof that a single gene was involved came from mapping the gene unambiguously to a single locus. Crossing also allows construction of strains with useful combinations of genes. By constructing diploids, we could show that the allele causing positive phototaxis showed simple dominance over the allele causing negative phototaxis. By inserting the *ey* gene, we could show that alteration of the eyespot does not necessarily affect the aggregation patterns caused by the two alleles of *agg-1*.

For constructing diploids we used a set of genetic markers that was sufficiently selective to reveal a genetic process not previously described in *Chlamydomonas*. This appears to be the meiosis of a zygote formed by fusion of four nuclei. Since this involves the mating of three individual cells, it should be possible with properly selective markers to develop a system for the routine mating of three strains. Such a system would confirm the meiotic diploid hypothesis and would provide a convenient method of testing complementation. It might also be a good way to study recombination of chloroplast genes. Chloroplast genes usually do not segregate in crosses because those from the mt^- parent are lost (Sager, 1972; Gillham, 1978). It is likely, however, that when two mt^+ parents contribute to a tetraploid zygote, chloroplast genes from both mt^+ parents survive.

Tetrad analysis is a powerful technique. Often, however, information contained in tetrad data is discarded because the analysis is not pushed to its limit. In our analysis of *agg-1*, we used the gamma distribution to predict genetic frequencies and the Monte Carlo method to apply chi-square statistics to small samples. We hope that this example will encourage the use of these and other computational techniques for extracting useful information from limited tetrad data.

We are grateful to Dr K. W. Foster and Dr G. W. Martinek for comments on the manuscript and to Dr H. C. Berg for the use of his PDP 11/34 computer. The work was supported in part by Public Health Service fellowship 4F01 GM 39082-03.

REFERENCES

Boscov, J. S. & Feinleib, M. E. (1979). Phototactic response of *Chlamydomonas* to flashes of light. II. Response of individual cells. *Photochemistry and Photobiology* **30**, 499–505.

Cobbs, G. (1978). Renewal process approach to the theory of genetic linkage: case of no chromatid interference. *Genetics* **89**, 563–581.

Cox, D. R. & Lewis, P. A. W. (1966). *The Statistical Analysis of Series of Events*. London: Chapman and Hall.

Ebersold, W. T. (1967). *Chlamydomonas reinhardi*: heterozygous diploid strains. *Science* (*Wash.*) **157**, 447–449

Ebersold, W. T. & Levine, R. P. (1959). A genetic analysis of linkage group I of *Chlamydomonas reinhardi*. *Zeitschrift für Vererbungslehre* **90**, 74–82.

Ebersold, W. T., Levine, R. P., Levine, E. E. & Olmsted, M. A. (1962). Linkage maps in *Chlamydomonas reinhardi*. *Genetics* **47**, 531–543.

Eves, E. M. & Chiang, K.-S. (1982). Genetics of *Chlamydomonas reinhardtii* diploids. I. Isolation and characterization and meiotic segregation pattern of a homozygous diploid. *Genetics* **100**, 35–60.

Fincham, J. R. S. & Day, P. R. (1965). *Fungal Genetics*, 2nd ed. Philadelphia: Davis.

Foster, K. W., Saranak, J., Patel, N., Zarilli, G., Okabe, M., Kline, T. & Nakanishi, K. (1984). A rhodopsin is the functional photoreceptor for phototaxis in the unicellular eukaryote *Chlamydomonas*. *Nature* (*Lond.*) **311**, 756–759.

Foster, K. W. & Smyth, R. D. (1980). Light antennas in phototactic algae. *Microbiological Reviews* **44**, 572–630.

Gillham, N. W. (1978). *Organelle Heredity*. New York: Raven Press.

Harris, E. H. (1982). Nuclear gene loci of *Chlamydomonas reinhardtii*. In *Genetic Maps*, vol. 2, (ed. S. J. O'Brien), pp. 168–174. Frederick, MD: National Cancer Institute.

Hartshorne, J. N. (1953). The function of the eyespot in *Chlamydomonas*. *New Phytologist* **52**, 292–297.

Haupt, W. (1959). Die Phototaxis der Algen. In *Handbuch der Pflanzenphysiologie*, vol. 17/1 (ed. W. Ruhland), pp. 318–370. Berlin: Springer-Verlag.

Hirschberg, R. & Stavis, R. (1977). Phototaxis mutants of *Chlamydomonas reinhardtii*. *Journal of Bacteriology* **129**, 803–808.

Hoshaw, R. W. (1965). Mating types of *Chlamydomonas* from the collection of Gilbert M. Smith. *Journal of Phycology* **1**, 194–196.

Huang, B., Ramanis, Z., Dutcher, S. K., Luck, D. J. L. (1982). Uniflagellar mutants of Chlamydomonas: evidence for the role of basal bodies in transmission of positional information. *Cell* **29**, 745–753.

Hudock, G. A. & Hudock, M. O. (1973). Phototaxis: isolation of mutant strains of *Chlamydomonas reinhardi* with reversed sign of response. *Journal of Protozoology* **20**, 139–140.

Hutner, S. H., Provosoli, L., Schatz, A. & Haskins, C. P. (1950). Some approaches to the study of the role of metals in the metabolism of microorganisms. *Proceedings of the American Philosophical Society* **94**, 152–170.

Kamiya, R. & Witman, G. B. (1984). Submicromolar levels of calcium control the balance of beating between the two flagella in demembranated models of *Chlamydomonas*. *Journal of Cell Biology* **98**, 97–107.

LEE, R. W., GILLHAM, N. W., VAN WINKLE, K. P. & BOYNTON, J. E. (1973). Preferential recovery of uniparental streptomycin resistant mutants from diploid *Chlamydomonas reinhardtii*. *Molecular and General Genetics* **121**, 109–116.

LEE, R. W., WHITEWAY, M. S. & YORKE, M. A. (1976). Recovery of sexually viable non-diploids from diploid *Chlamydomonas reinhardtii*. *Genetics* **83**, s44.

LEVINE, R. P. & EBERSOLD, W. T. (1960). The genetics and cytology of *Chlamydomonas*. *Annual Review of Microbiology* **14**, 197–216.

LEWIN, R. A. (1952). Ultraviolet induced mutations in *Chlamydomonas moewusii* Gerloff. *Journal of General Microbiology* **6**, 233–248.

MARTINEK, G. W., EBERSOLD, W. T. & NAKAMURA, K. (1970). Mitotic recombination in *Chlamydomonas reinhardi*. *Genetics* **64**, s41–2.

MATAGNE, R. F. & ORBANS, A. (1980). Somatic segregation in diploid *Chlamydomonas reinhardii*. *Journal of General Microbiology* **119**, 71–77.

MOREL-LAURENS, N. M. L. & FEINLEIB, M. E. (1983). Photomovement in an eyeless mutant of *Chlamydomonas reinhardtii*. *Photochemistry and Photobiology* **37**, 189–194.

NULTSCH, W., THROM, G. & VON RIMSCHA, I. (1971). Phototaktische Untersuchungen an *Chlamydomonas reinhardii* Dangeard in homokontinuierlicher Kultur. *Archiv für Mikrobiologie* **80**, 351–369.

PERKINS, D. D. (1953). The detection of linkage in tetrad analysis. *Genetics* **38**, 187–197.

SAGER, R. (1955). Inheritance in the green alga *Chlamydomonas reinhardi*. *Genetics* **40**, 476–489.

SAGER, R. (1972). *Cytoplasmic Genes and Organelles*. New York and London: Academic Press.

SCHAECHTER, M. & DELAMATER, E. D. (1956). Studies on meiosis in *Chlamydomonas*. *Journal of the Elisha Mitchell Scientific Society* **72**, 73–80.

SMITH, G. M. & REGNERY, D. C. (1950). Inheritance of sexuality in *Chlamydomonas reinhardi*. *Proceedings of the National Academy of Science, USA* **36**, 246–248.

SMYTH, R. D. & BERG, H. C. (1982). Change in flagellar beat frequency of *Chlamydomonas* in response to light. *Cell Motility Supplement* **1**, 211–215.

SMYTH, R. D., MARTINEK, G. W. & EBERSOLD, W. T. (1975). Linkage of six genes in *Chlamydomonas reinhardtii* and the construction of linkage test strains. *Journal of Bacteriology* **124**, 1615–1617.

SMYTH, R. D. & EBERSOLD, W. T. (1970). A *Chlamydomonas* mutant with altered phototactic response. *Genetics* **64**, s62.

STRASBURGER, E. (1876). Wirkung des Lichtes und der Wärme auf Schwarmsporen. *Jenaische Zeitschrift für Naturwissenschaft* **12**, 551–625.

WARR, J. R. (1968). A mutant of *Chlamydomonas reinhardii* with abnormal cell division. *Journal of General Microbiology* **52**, 243–251.