A STUDY OF THE SEXUALITY AND GENETICS OF *EUDORINA ELEGANS* EHRENBERG

by

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B.A., University of California, Riverside, 1969

THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
in the Department
of
Biological Sciences.

SIMON FRASER UNIVERSITY
May, 1974

Approval

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Degree: Master of Science

Title of Thesis: A Study of the Sexuality and Genetics of *Eudorina elegans* Ehrenberg

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Abstract

Sexual reproduction of Eudorina elegans was investigated in order to learn to control the sexual cycle. Gametogenesis is induced by a proper balance of nutrients in a minimal medium -- namely calcium and nitrogen -- while an added carbon source has no effect on gamete induction. Forty-eight hours of a standard light/dark cycle at 32° in Volvox medium is necessary for gametogenesis. However, "conditioned" medium does induce sperm production in only 24 hours under the same conditions suggesting a hormonal induction system. Zygote formation is also dependent on nitrogen and calcium concentrations and will not proceed in darkness. Germination of zygotes remains inconsistent even though a number of germination methods were tried.

Differentiation of male and female vegetative organisms was attempted through immunological means using cellular and flagellar antigens. Immunological studies utilized agglutination tests, immobilization tests, and agar diffusion methods. No differentiation of male and female colonies could be detected in Eudorina using either flagellar or cellular antisera.

Genetic analysis of E. elegans utilized 14 various

mutants that had been isolated by UV mutagenesis. Upon crossing these mutants with the wildtype strain, all of the markers exhibited Mendelian inheritance and were found to assort independently from the sex alleles. Data from mutant-mutant crosses were used to construct a linkage map. The light green mutants mapped at several different loci, while the 2 very pale mutants showed close linkage. Sexual compatibility varied considerably among the various types of mutants and proved to be a limiting factor in obtaining data from mutant-mutant crosses for the construction of a complete linkage map.

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Acknowledgements

To Dr. C. L. Kemp, who has been both supervisor and friend,

to Dr. J. R. Stein for her encouragement, advice, and critical evaluation of this thesis,

to Dr. I. R. Glen for his help with certain immunological techniques and his constructive criticisms,

to Mr. Ken Lee for his advice and encouragement in the laboratory,

to Miss Gina Bennett and Miss Anne Toby for their invaluable technical assistance, and

to Mrs. Donna Wilson for her patience in typing this thesis,

I express my sincere gratitude.

A STUDY OF THE SEXUALITY AND GENETICS OF *EUDORINA ELEGANS* EHRENBERG

CHAPTER I

INTRODUCTION

Eudorina elegans Ehrenberg appears to have potential as a system for the study of sexuality, genetics, and morphogenesis in lower plants. Being a green, colonial, heterothallic alga belonging to the order Volvocales, this organism is simple in form, yet it does have a sexual cycle. Because of its short life cycle and growth characteristics, techniques developed for bacterial studies can be adapted to studies of *Eudorina*. Considering these advantages, a series of experiments were initiated to study the physiology of sexual reproduction and the genetics of such a primitive organism and to speculate on the genetic regulation involved in such processes.

Previous studies of sexuality and genetics of *Eudorina* have included speciation and sexual compatibility of *Eudorina* populations including descriptions of

mating behavior and zygote germination of various species (Goldstein, 1964). Recent investigations (Szostak, Sparkuhl and Goldstein, 1973) suggest that a hormone may regulate sexual induction. A fundamental genetic investigation of Eudorina was attempted using drug resistant mutants (Mishra and Threlkeld, 1968). This study succeeded in establishing that genetic analysis was possible and that uniparental as well as Mendelian inheritance occurs in E. elegans.

Studies involving zygote formation and germination of E. elegans have usually utilized Goldstein's methods (Goldstein, 1964), however, my preliminary experiments gave variable results with, almost always, a low percentage of germination. Therefore, the necessity of examining the physiological aspects of sex, in order to control the sexual cycle, became apparent. Results would then be predictable and genetic analysis would be practicable. Chapter II deals with the physiology and parameters of sexual reproduction of E. elegans.

Another problem that arose upon preliminary investigation of Eudorina was the necessity to differentiate male and female organisms. The methods involving growing of isolates and testing each for its ability to produce sperm or to form zygotes with appropriate

testers are very tedious. Attempts were made to improve and simplify the differentiation using serological techniques. These studies are presented in Chapter III.

Chapter IV describes the actual genetic analysis of E. elegans. Chlorophyll deficient mutants, a few morphologically abnormal coenobial strains, and a nicotinamide-requiring alga have been isolated in our laboratory (Kemp, unpubl.). These mutants have been crossed with the wild type strain as well as crossed with each other in order to learn how these characteristics are inherited. Linkage maps are constructed where possible and sexual compatibility of mutants is discussed.

Chapter V presents an overall discussion of the evolutionary significance of the results of this investigation of sexuality and genetics of Eudorina elegans.

CHAPTER II

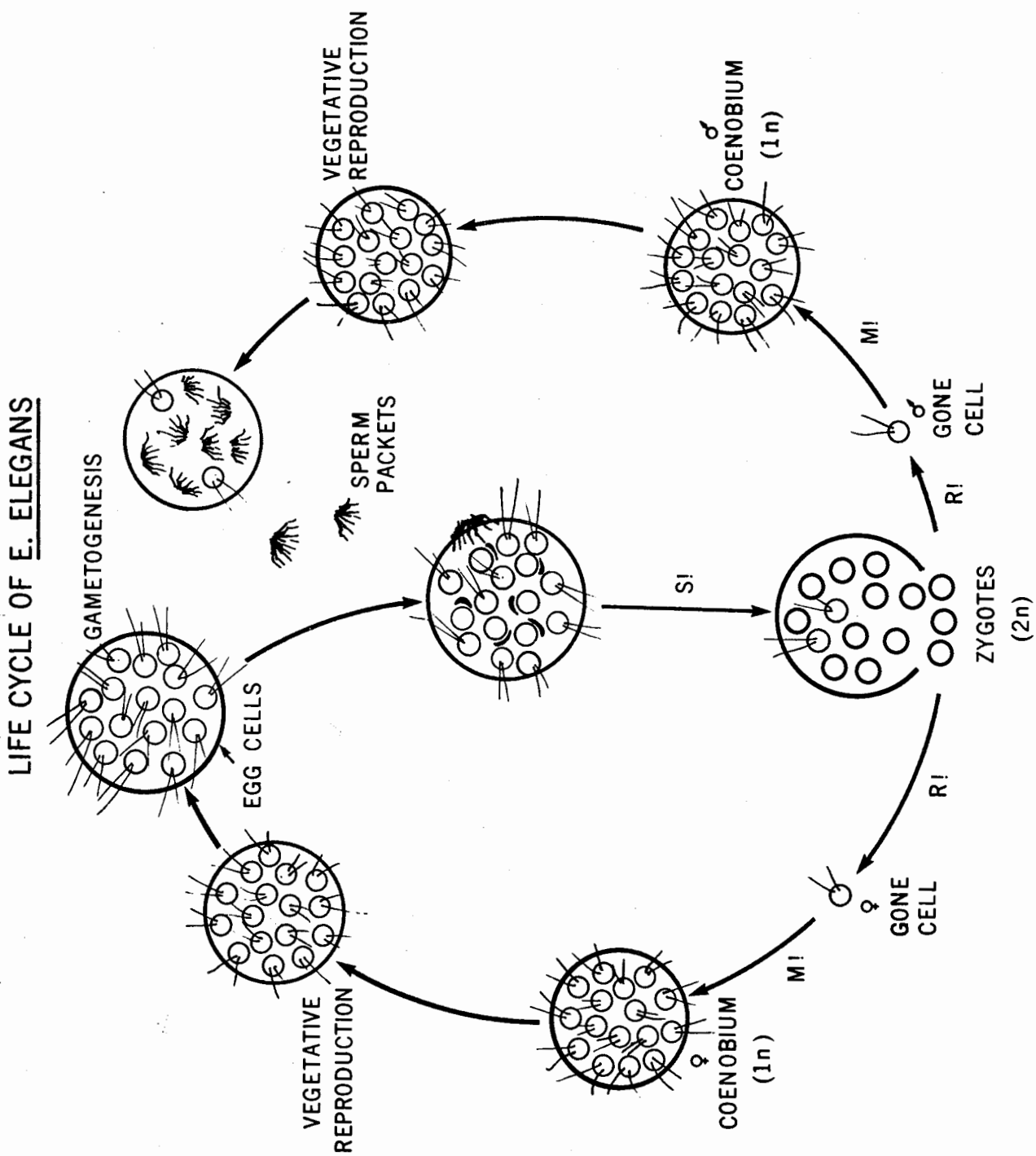
SEXUAL PHYSIOLOGY OF EUDORINA ELEGANS

INTRODUCTION

Eudorina elegans has a very simple life cycle as illustrated in Figure II-1. The 16- or 32-celled coenobium grows vegetatively until gametogenesis occurs. Then cells in a male coenobium develop into 32-celled sperm packets. There is no apparent differentiation of the female cells. Free-swimming sperm packets are attracted to the female coenobia where these packets break down. Sperm enter the female coenobial matrix separately and eventually fertilize the female cells. A thick walled zygote forms which remains dormant until germination occurs at which time a single gone cell is released.

Conditions necessary for gametogenesis, zygote formation, and germination are not specifically known for Eudorina elegans, however, a number of papers have been published concerning control of sexuality in Chlamydomonas. Nutritional control of sexuality (Sager and Granick, 1954) as well as the effect of light and dark periods (Lewin, 1956) have been investigated. Some comparative studies of sexual reproduction with

Figure II-1


LIFE CYCLE OF E. ELEGANS
GAMETOGENESIS
EGG CELLS
VEGETATIVE REPRODUCTION
♀ COENOBIUM (1n)
M!
♀ GONE CELL
R!
ZYGOTES (2n)
S!
♂ GONE CELL
R!
♂ COENOBIUM (1n)
M!
VEGETATIVE REPRODUCTION
SPERM PACKETS

several Chlamydomonas species have been done (Trainor, 1959; Wiese and Jones, 1963). Physiological aspects of reproduction of Platydorina are discussed by Harris and Starr (1969). These papers, as well as those by Starr's group on hormonal induction of sexuality in Volvox (Darden, 1966; Kochert, 1968; McCracken and Starr, 1970; and Vande Berg and Starr, 1971), show that conditions for sexual reproduction are not the same for all green colonial or unicellular algae. Conditions even differ among species of Chlamydomonas and species of Volvox. Therefore, one cannot assume that physiological conditions are similar for Eudorina and, for instance, Platydorina.

Because of the variability obtained in the initial attempts at sexual induction, zygote formation, and germination of Eudorina using Goldstein's procedure (1964), it was apparent that a detailed study of sexuality was necessary before genetic analysis on any scale could proceed.

MATERIALS AND METHODS

A. Organisms

The cultures of E. elegans, 1200 (male) and 1201 (female), were obtained from the Culture Collection of Algae at Indiana University, Bloomington, Indiana

(Starr, 1964). These strains, which were originally designated 62m and 62f respectively, were isolated and described by Goldstein (1964).

B. Media and Culture Conditions

Liquid cultures were grown in modified Bristol's salt solution (Cain, 1964), denoted BM, or in enriched medium (BC) made by adding 200 ml of a modified Euglena medium to 800 ml of BM (Kemp and Wentworth, 1971). Gamete formation was initially induced in soil extract (SE) medium (Mishra, 1967). Volvox medium (VM) (Darden, 1966), from which biotin and vitamin B12 were omitted, was eventually used instead of SE because of its known composition and ability to induce sexuality. The composition of all media are presented in Appendix I.

Solid medium appropriate for stock maintenance or for plating germinating zygospores was obtained by adding 1.0 to 1.5% w/v Difco agar to BC medium. Top agar for the pour-plating technique was made by adding 0.075 to 0.6% Difco agar to BC medium. All media were sterilized by autoclaving at 15 p.s.i. for 15 minutes.

Conditioned medium, CM, was obtained by growing a culture of Eudorina in VM for various periods of time under standard conditions at 32°C. The culture was first centrifuged and then the supernatant was filtered

through a Millipore filter to eliminate any cellular debris.

Stock cultures were maintained axenically in flasks of BM and on BC agar slants. Liquid cultures were grown on a shaking platform at 22 ± 1°C or 32 ± 1°C with a standard 16:$\overline{8}$ light:dark cycle. Illumination of 1000 ft-c was provided by standard cool white fluorescent lights. The liquid cultures were transferred weekly. Cultures on slants were transferred every 4 to 6 weeks.

C. Procedure

The basic method for sexual reproduction, as described by Goldstein (1964), was first employed. The male and female strains were grown separately in SE at 20°C for 3-4d. Two-3 ml of each mating type were mixed in watch glasses supported on glass triangles in petri dishes and an equal amount of fresh SE was added. A 5% solution of $NaHCO_3$ was added to the bottom of the petri dish. The watch glasses were illuminated at 250-350 ft-c. Zygotes formed within 1-4 d.

Goldstein employed 2 methods for zygote germination. Method I: the resulting zygotes in the watch glasses remained in the light for 5-7 d and changed color from

dark green to orangish-brown; the zygotes were removed from the watch glasses with a pipette and spread over the surface of 1% SE agar plates; each plate was inverted over a petri dish of chloroform for 30 sec to kill vegetative cells; the plates were incubated at 37°C for 2-3 d, then returned to 250-350 ft-c illumination at 20°C. Method II: the resulting zygotes matured under illumination of 250-350 ft-c for 7-10 d; the dishes of zygotes were then placed in the dark at room temperature for 1 week and allowed to dry; the zygotes were rewet with SE and placed in the light at room temperature; after 2-3 d the initial supernatant was replaced with fresh medium. Germination generally occurred within 24 hours.

In examining sperm formation and zygote production, practically all conditions were varied in an effort to achieve optimum conditions. The parameters investigated include temperature (22°C vs 32°C), light (0-1000 ft-c), addition of a carbon source (0.9-3.6 mM of sodium acetate) to minimal salts medium, the utilization of various media (SE, BM, BC, VM), nitrogen levels (0-1.16 x 10^{-2} M), calcium levels (1.3 x 10^{-4} M to 1.0 x 10^{-3} M), and the use of CM to examine the possibility of a hormonal effect on sexual induction.

In addition to the above parameters, the necessity for the addition of fresh medium to the mating mixture and the presence of the $NaHCO_3$ solution was examined with regards to zygote production. The influence of the age of growing cultures prior to and during sexual induction was examined to see if any physiological induction of the female strain was necessary and how long the male culture would remain sexually induced.

Factors influencing germination were also tested. Light maturation periods were varied from 0-10 d. Light intensity during this period was also varied from 50-1000 ft-c. The necessity and extent of the 37°C temperature shock was examined as well as the usefulness of different "shocking" methods to induce germination. These methods involved prolonged exposure to 4°C, -15°C, and ultraviolet light as well as an increase in incubation temperature to 60°C prior to rehydration. The percent of agar (.075 to 1.5%) and the type of media (SE, BM, BC, VM) used for germination were also varied. The importance of washing the zygotes or replacing the medium after 24 hours in order to induce germination was also examined.

Sperm production and zygote formation were scored as present or absent using microscopic examination of

the cultures. Sperm clump and coenobial counts were made on a Fuchs-Rosenthal Haemocytometer. An estimate of the germination efficiency was obtained from the fraction of empty (germinated) zygospores on the bottom of the watch glasses.

If zygotes were kept for any length of time, they were dried in the mating dish or test tube and stored in the dark at room temperature.

RESULTS

A. Gametogenesis

Several factors were found to influence or improve gametogenesis. Sperm induction was quite variable at 22°C whereas at 32°C sperm packets were almost always present in VM within 2d and in SE in 2-3d. At least 48 hrs of a 16:$\overline{8}$ cycle at 32°C are necessary for sperm formation. Table II-1 shows no sperm packets in flasks of VM kept in total darkness or with only 24 hrs incubation in the standard light cycle. The addition of Na acetate did not replace the necessity of light in sexual induction. At all Na acetate concentrations sperm packets were present in cultures maintained at 32°C in the standard light cycle for 3 d, while none were present in those flasks kept in the dark at 32°C.

The type of media used and the nutrient levels

Table II-1

Effects of Light on Sexuality

Cultures were grown in flasks of VM on a shaker at 32°C under a standard 16:$\overline{8}$ cycle or in total darkness.

To test zygote formation 1200 and 1201 were grown under similar conditions before mixing. Watch glasses were used for mating mixtures.

All variations were tried at least twice.

Table II-1

#	Growth Conditions	1200 after 3 d	1200-1201 mixture after 7 d in:	
			light cycle	darkness
1	3d light cycle	many sperm	zygotes	no zygotes
2	3 d darkness	no sperm	-	-
3	24 hr light cycle, 48 hrs darkness	no sperm	-	-
4	48 hrs darkness, 24 hrs light cycle	no sperm	-	-
5	3d light cycle, 3.6mM Na acetate	few sperm	zygotes	no zygotes
6	3d light cycle, 1.8mM Na acetate	many sperm	zygotes	no zygotes
7	3d light cycle, 0.9mM Na acetate	many sperm	zygotes	no zygotes
8	3d darkness, 3.6mM Na acetate	no sperm	-	-
9	3d darkness, 1.8mM Na acetate	no sperm	-	-
10	3d darkness, 0.9mM Na acetate	no sperm	-	-

seem quite important for gametogenesis. SE is a fairly good medium for sperm induction, however, since it is not a defined synthetic medium, the levels of nutrients necessary for gametogenesis cannot be tested. BM and BC support vegetative growth of Eudorina very well, but only rarely are sperm packets formed in these media. VM, on the other hand, supports gametogenesis very well and gives even more consistent results than SE. The composition of VM and BM were consequently compared. If the levels of Ca^{++} and NO_3^- in VM, 5×10^{-4}M and 1×10^{-3}M respectively, were changed to those in BM, 2.5×10^{-4}M of Ca^{++} and 2.9×10^{-3}M of NO_3^-, no sperm were formed. If these ions in BM were changed to the levels in VM, good sperm induction resulted. Table II-2 shows the effects of increasing and decreasing the NO_3^- and Ca^{++} concentrations in BM. A certain amount of Ca^{++} is definitely necessary for gametogenesis. A decrease in NO_3^- also induces sperm production. There also seems to be an inter-relationship between these two nutrients in their effect on sperm production. As the Ca^{++} is increased, so can the NO_3^- be increased while still allowing sperm production.

Conditioned medium (CM) also seems to increase sperm formation. Table II-3 shows that medium in which

Table II-2

Effects of NO_3^- and Ca^{++} on Sexuality

-- Ca^{++} and NO_3^- varied in BM. Cultures were grown in flasks on a shaker at 32°C.
-- 1200 was checked for sperm packets at 24, 48 and 72 hrs.
-- To test for zygote formation, 1200 and 1201 were grown under similar conditions for 3 d before mixing and were checked for zygotes 10 d later.

All variations were tried at least twice.

♂ - few sperm
♂♂ - many sperm
Z - few zygotes
ZZ - many zygotes
G < 10% germination
GG > 10% germination

Table II-2

NO_3^- level

Ca^{++} level	0	$(1/100)$* .00003M	$(1/50)$ .00006M	$(1/4)$ .00075M	$(1/2)$ .0015M	(1) .0029M	(2) .0058M	(4) .0116M
$(1/2)$* .00013M	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -
(1) .00025M	24 hrs ♂ 48 hrs ♂ Z -	48 hrs ♂♂ Z -	48 hrs ♂♂ ZZ -	48 hrs ♂♂ ZZ GG	48hrs ♂♂ ZZ -	- - -	- - -	- - -
(2) .0005M	24 hrs ♂ 48 hrs ♂♂ - -	24 hrs ♂ 48 hrs ♂♂ Z -	24 hrs ♂ 48 hrs ♂♂ - -	24 hrs ♂ 48hrs ♂♂ ZZ GG	48 hrs ♂♂ ZZ GG	48 hrs ♂♂ ZZ -	- - -	- - -
(4) .0010M	48 hrs ♂♂ - -	48 hrs ♂♂ Z G	48 hrs ♂♂ ZZ -	48 hrs ♂♂ ZZ G	48 hrs ♂♂ ZZ G	48 hrs ♂♂ ZZ GG	48 hrs ♂♂ - -	- - -

* Concentration of ions with respect to BM

Table II-3

Effect of CM on Sperm Induction

All CM is obtained from cultures grown in VM under 16:$\overline{8}$ cycle (note exception CM_h).

Five ml of CM were placed in a small flask with 5-10 x 10^3 coenobia/ml (no.1-4) or 2-4 x 10^4 coenobia/ml (no.5-10) of male Eudorina coenobia.

Table II-3

no.	CM	CM from culture of	Sperm packets:coenobia after 24 hrs at 32°C	Sperm packets:coenobia after 24 hrs at 22°C	Sperm packets:coenobia after 72 hrs at 22°C
1	CM_a	1200 5d at 22°C	6:5	1:2	
2	CM_b*	1200 5d at 22°C	3:1	1:3	
3	CM_c	1200 5d at 32°C	5:3	1:2	
4	Control	fresh VM	1:2	< 1:200	
5	CM_d	1200 6d at 32°C	1:4		2:15
6	CM_e	1200 10d at 32°C	1:3		1:12
7	CM_f	1200 14d at 32°C	1:2		1:16
8	CM_g	1201 6d at 32°C	1:7		I:16
9	CM_h	1200 6d at 32°C in darkness	<1:100		1:93
10	Control	fresh VM	1:11		1:50

* no sperm present in culture from which CM is obtained.

the male strain of Eudorina grew at least 5 days does significantly increase sperm formation compared to control cultures in fresh VM. Light is still necessary for gametogenesis as shown by CM_h. Whether CM_g, made from growing the female strain in VM, has any effect on sperm induction is still somewhat questionable. At 32°C where there is generally much less variability than at 22°C, more sperm packets are induced in CM_d, in which the male strain had grown, compared to CM_g, in which the female strain had grown. However, CM_g does induce more sperm than present in the control with fresh VM.

Several observations concerning the age of the cultures used for sexual induction have been made. If the male strain is inoculated directly into VM from solid medium without growing at least a few days in liquid beforehand, sperm production is negligible. The same is true, if the 1200 culture is taken from a liquid culture 2 weeks old or more. The best sperm production comes from a culture approximately 2-7 d old before inoculation into VM. The type of medium used to originally grow the cultures before inoculation into VM had no effect on sexual induction. Thorough washing of the cells did not increase sexual induction

either. Incubation on a shaking platform rather than sitting on a shelf, did increase sperm formation, possibly because the generation time is then decreased.

There is still the question of female sexual differentiation in E. elegans. Can vegetative cells in 1201 act as egg cells at any time or is there some physiological differentiation that must take place? This question remains unresolved. Cultures of 1201, grown in either BM or BC, if washed only once, and then placed in a mating dish with 1200 and VM, could still act as eggs, and zygotes would be formed. However, observations showed only the larger coenobia were fertilized. The smaller and more active coenobia never seemed to attract the sperm clumps. A few large coenobia are always found in BM and BC, but if NO_3^- is increased 2 or 4 times the amount in BM, only the very small active coenobia are present and no zygotes are formed after mixing with 1200. Again, an initial fresh liquid culture is a necessity for good zygote production.

B. Zygote Formation

Factors affecting zygote formation proved to be similar to those affecting gametogenesis. Mated cultures at 32°C gave more consistent results than those incubated at 22°C. Watch glasses in total darkness, with or

without the addition of Na acetate, produced no zygotes, while those in the light did, as shown in Table II-1. Varying the light intensity from 50 to 1000 ft-c made very little difference on the ability to produce zygotes, but a high yield was usually obtained at 1000 ft-c. The presence of $NaHCO_3$ solution in the bottom of the petri dish seemed to enhance zygote formation, but was certainly not necessary. Zygotes were also formed in test tubes, flasks, and watch glasses with nothing, or only water, in the petri dish. The addition of fresh medium in the mating dish did not enhance zygote yield. This step was eliminated in other experiments.

There is good zygote production in VM and SE, however, no zygotes are produced in $PO_4^{\equiv}$ buffer or BC, and only rarely in BM, no matter in what medium the strains are sexually induced. Cultures grown in BM or BC just prior to mating form zygotes in VM or SE, but sexual induction must then occur in the mating vessel. Table II-2 shows the effect of Ca^{++} and NO_3^- concentrations on zygote formation. As expected, the results resemble those for sperm induction, however, there are differences. At NO_3^- concentrations less than 6×10^{-5}M, zygotes are very few in number and often morphologically abnormal. Vegetative cells do not remain viable at those concen-

trations and the conclusion is that some nitrogen is necessary for good zygote formation. There seems to be a maximum NO_3^- level for zygote production. Even though sperm are produced at 5.8×10^{-3} of NO_3^- and 1×10^{-3}M of Ca^{++}, no zygotes are formed. This result is possibly due to the physical state of the female strain since at this concentration all female coenobia are very small and active. As noted earlier, this type of coenobium never seems to be fertilized by sperm.

The age of the sexually induced cultures before mixing them together has little effect on the ability to produce zygotes. Even cultures as old as 14 d will usually produce a few zygotes. However, the best zygote yield is obtained if both cultures are from 2 to 4 d old before mixing.

C. Zygote Germination

Zygote germination remained extremely variable. The percentage of germination in liquid varied from less than 1% to over 90%, with an average range of 5% to 20%.

A light maturation period of 4-7 d after zygote formation is definitely necessary for germination. Attempts to shorten the light maturation period to 0-3 d failed to increase germination. In fact, in 24 out of

32 watch glasses, no germination occurred at all. The light intensity during this period was varied from 50 to 1000 ft-c, but it did not affect germination. However, at low light intensities the zygotes remained dark green rather than turning orangish-brown during the light maturation period. But after several days in the dark, all the zygotes turned brown in color. The importance of the color change in the zygote for meiosis and germination is not known.

A dark period at room temperature seems to have no effect on zygote germination. However, a period of at least 48 hrs at 37°C (in the dark) is quite necessary. This incubation period did not usually yield greater than 20% germination, but rather insured that some germination occurred. Increasing the time at 37°C even up to 10 d, had no harmful or enhancing effects on the percent germination. Usually germination for genetic analysis was preceded by a dark period at room temperature of approximately 7 d followed by incubation at 37°C for 5-7 d. These steps insured that the mating culture was completely dried and all of the vegetative cells were killed. If these steps were shortened, germination would still occur, but vegetative cells sometimes remained viable. These steps also eliminated the necessity of

using chloroform vapor to kill vegetative cells and possibly kill the zygotes as well.

In order to increase percent germination, attempts were made to shock the zygotes into germinating. Zygotes were frozen at -15°C as well as kept at 4°C from 1-14 d with no change in the yield of gone cells. Zygotes were also exposed to UV light at doses of 600 and 1000 ergs/mm^2, but very little germination, if any, occurred thereafter. All these variations were tried in combination with a dark period at room temperature, at 37°C, or by going directly into the light. None of these variations increased germination significantly.

Increasing the incubation temperature from 37°C to 60°C was also tested as a possible mode of increasing the number of zygotes that germinate. The increase in temperature to 60°C for 24, 48, or 72 hrs does induce more zygotes to germinate (usually greater than 50% were induced), however, very few gone cells remained viable. They seemed to be aborted from the zygospore and die before becoming mobile. An incubation temperature of 37°C to 40°C seems to give best results for induction of germination and gone cell viability.

After incubation at 37°C the zygotes are flooded with liquid medium and placed in the light. If the

medium is changed for fresh medium in 24 or 48 hrs, germination is assured to take place the following day. On a second or third change in medium no further germination occurred. The type of medium used made little difference; BM, BC, SE, and VM gave similar results. A temperature of 22°C or 32°C in the light made no difference in germination. Zygotes that were initially washed 3 or 4 times still needed incubation in light and a change of fresh medium after one day to induce germination. Rarely a zygote will germinate without the change of medium, but one is assured of a greater number germinating at the same time if the medium is replaced.

BC liquid medium and various percentages of agar in BC solid medium were compared for their effect on germination. Very little germination, if any at all, took place on 1% or 1.5% agar, but increasing frequencies of germination occurred as the agar in the top agar decreased from 0.6 to 0.075%. The actual percent germinating was still quite low and variable. If dried zygospores were first flooded with BC, incubated in the light for 24 hrs, and then plated in 0.6% or less top agar, germination was just as good as germination in liquid.

DISCUSSION

A. Gametogenesis

Sexual induction is consistent if young, actively growing cultures of *E. elegans* are grown in VM at 32°C for at least 48 hrs of a 16:8 cycle. Nutrient levels of Ca^{++} and NO_3^- seem important in gametogenesis. The requirement for nitrogen depletion is not all that surprising. Sager and Granick (1954) found the absence of nitrogen necessary for gametogenesis in *Chlamydomonas reinhardi*. Trainor (1959) found that with a decrease in NO_3^-, there was an increase in yield of zygotes in 3 of 4 chlamydomonad species tested. Harris and Starr (1969) also found a linear relationship between a decrease in nitrogen and an increase in sperm production in *Platydorina*. This type of metabolic starvation or malnutrition does seem to initiate the sexual cycle in *E. elegans*. However, there is not an inverse linear relationship of nitrogen level and sexual induction as is true for *Chlamydomonas* or *Platydorina*. At very low levels (less than 3×10^{-5}M) and in the absence of NO_3^- where vegetative growth is definitely hindered, sperm production is limited as well. Sperm are usually seen one day sooner than at the higher more optimal NO_3^- concentrations. These data seem to coincide with results

obtained using the non-motile Golenkinia (Ellis and Machlis, 1968), where only the onset of sperm production and not the sperm yield was found to be inversely proportional to the nitrogen level. The optimum amount of sperm production in Golenkinia was achieved at about 1×10^{-3}M of NO_3^- in the medium.

A minimum amount of Ca^{++} is necessary for sexual induction. Ca^{++} has been found to be a requirement for fertilization in Golenkinia (Ellis and Machlis, 1968) and Chlamydomonas (Wiese and Jones, 1963) but not for gametogenesis.

The fact that by increasing the Ca^{++}, the nitrogen level can also be increased, while giving the same result in the male strain, is somewhat puzzling. One can only speculate on the role of calcium and nitrogen in sexual induction in Eudorina. Possibly sexual differentiation is not directly induced by nitrogen depletion in E. elegans, as in Chlamydomonas. A decrease in nitrogen may initiate production of a hormone substance, that also requires Ca^{++}, which in turn will induce sperm production in E. elegans. However, the interaction of calcium and nitrogen might be better explained by a competitive mechanism. There may be two different modes of action for sexual induction in E. elegans. Either a sufficient

amount of Ca^{++} is required, possibly for a large amount of inducing hormone, in order for sperm to develop, or only the minimum amount of Ca^{++} (2.5×10^{-4}M) for the minimum amount of inducing substance along with a decrease in NO_3^- is necessary for gametogenesis.

The idea of a hormone inducing sexuality does not merely come from the observed Ca^{++}-NO_3^- interaction. In the past ten years, Starr and his group (reviewed in Starr, 1968, and Darden, 1970) have studied hormonal regulation of sexual differentiation in Volvox species. More recently, Goldstein and his students (Szostak, et al., 1973) have published a paper suggesting a hormone system in E. elegans. Their data, and my preliminary investigation with CM as outlined in Table II-3, shows sperm production is initiated, or at least enhanced, by growth in CM. This result might suggest that sperm are induced by depleting nutrients, or more specifically, by depleting nitrogen, in CM due to the previous algal growth. However, if that were true, results in Table II-2 should show better sperm production at lower concentration of NO_3^- but that is not the case. Also, a hormone system for the male strain might explain the observation of such variability in the number of sperm produced. Even though sperm are present in VM after 48 hrs at

32°C the number may vary considerably. Possibly, the amount of hormone present is different in these instances.

Factors other than a possible hormone or balance of nutrients are still necessary for gametogenesis since growth in the presence of CM does not assure sperm production. CM_b, (see Table II-3) obtained from a 5 d culture of 1200 in VM at 22°C, had no observable sperm present at the time it was harvested. But CM_b did enhance sperm induction when tested showing the inducing substance was present. CM_h shows that light is still a necessity for production of sperm even in the presence of CM.

The timing mechanism for gametogenesis is not clearly established. According to Szostak, 2 generations are necessary -- possibly one generation for hormone production, the second generation for cellular differentiation (Szostak, et al., 1973). Considering a generation time of a little over 24 hrs for the 1200 strain at 32°C (Kemp, pers. comm.) and since at least 48 hrs in a 16:$\overline{8}$ cycle is necessary for sperm production at 32°C (Table II-1), the logical conclusion is that light is necessary for both processes -- hormone production and sperm production. If a culture is incubated for 24 hrs under standard light conditions and then placed in the dark, or kept first in the dark for 24 hrs or more

and then incubated under standard light conditions for 24 hrs, the result is the same -- no sperm are produced. The idea that 2 modes of action may induce sexuality in E. elegans, either a large amount of hormone or a depletion of nitrogen, would explain the phenomenon of occasional sperm present at 24 hrs at low NO_3^- levels (due to nitrogen depletion) and sperm in 48 hrs at high NO_3^- and Ca^{++} levels (due to a large amount of hormone).

B. Zygote Production

Zygote production is very good and quite consistent, if 2-3 d old cultures of 1200 and 1201 grown in VM are mixed and incubated at 32°C. Zygotes will form in 1-2 d if incubated under standard light conditions. Light for zygote formation is also necessary for other algae (Platydorina, Harris and Starr, 1969; and Chlamydomonas, Trainor, 1959) with the exception of Chlamydomonas reinhardi which can produce zygotes in the dark if certain amounts of acetate and NO_3^- are present (Sager and Granick, 1954).

The necessity for calcium and nitrogen in zygote production in E. elegans is apparent, however, it is not clear whether these requirements affect zygote formation and/or female differentiation. There is a definite inhibitory NO_3^- level, namely 5.8×10^{-3}M, which inhibits both enlargment of female cells in Eudorina and zygote formation.

Tsubo (1956), using NH_4NO_3, observed that Chlamydomonas sp. 24 gametes will not copulate in the presence of assimilable nitrogen. Ca^{++} is necessary for zygote production in other green algae as well. A minimum level of 1×10^{-3}M of Ca^{++} is necessary for fertilization in Golenkinia (Ellis and Machlis, 1968). Wiese and Jones (1963) reported that Ca ions are necessary for flagellar agglutination in 3 Chlamydomonas species mating activity but not for the formation of the cytoplasmic bridge between mating gametes.

C. Zygote Germination

Germination is best obtained if zygotes, which are matured in the light for 5-8 d, are dried and incubated at 37°C in the dark for at least 3 d. If the zygotes are flooded with medium and incubated in the light and the medium is replaced in a day or so, germination occurs within 24 hrs. Germination seems quite dependent on the change of fresh medium. Possibly the change is necessary to get rid of an inhibitory substance preventing germination that is not washed away prior to the first flooding because of either being bound to the zygote or being produced after the first flooding occurs.

Even this method does not assure a high yield of germinating zygotes. There is still the question as

to why such a low percentage of germination. Possibly the optimum nutritional requirements are not yet met during zygote formation even though literally thousands of zygotes per ml can be formed. Most of these zygotes may simply not be viable, though this explanation does not account for such variability in germination. Germinating efficiency may be determined genetically or developmentally which might always give a variable yield of gone cells. Chaing and his co-workers (Chaing, Kates, Jones and Sueoka, 1970) have found that the mating behavior of stocks of Chlamydomonas reinhardi obtained from the Indiana Culture Collection was rather unstable. The mating efficiency of subclones from particular stocks varied considerably. Kemp (pers. comm.) found that 1192 and 1193, 2 E. elegans strains reported as compatible from the Indiana Culture Collection, had lost their sexual cycle completely. Germinating ability in E. elegans may be as variable and as easily lost as the mating ability in these other stock cultures.

CHAPTER III

SEROLOGY OF *EUDORINA ELEGANS*

INTRODUCTION

In anticipation of genetically analyzing *E. elegans*, there was the problem of differentiating male and female gone cells. The production of sperm packets by the male strain allows microscopic differentiation of appropriately induced isolates. However, even growing the separate clones in VM or SE, not all males will induce sperm production at the same time. It is usually necessary to employ a second method for sexual differentiation - that of mating a known male and female strain with the unknown clonal isolate in separate watch glasses or spot plates. If the isolate is female, zygotes will form with the male strain. If the isolate is male, zygotes are produced with the female strain. The number of isolates that must be analyzed and the limited facilities make both methods tedious and time consuming.

Serology is an important technique in differentiating bacterial strains which are very similar metabolically and morphologically. For example, a simple agglutination test will distinguish *Streptococcus* Group A from *Streptococcus* Group C. If an antigen-antibody test could be utilized in distinguishing male and female algal cells,

the process of sexual differentiation would be greatly simplified.

Theoretically, the use of serological techniques seems quite feasible. The production of antibodies is very sensitive in response to the presence of antigens. Antigens which vary only in one carbohydrate or polypeptide chain can be distinguished from each other by the difference in the antibodies formed. Thus, the male and female strains of E. elegans might be different enough on the molecular level as to be distinguishable by an antigen-antibody reaction.

The application of serology is not new to the field of Phycology. Brown used immunological studies to compare the Chlorococcales genera Tetracystic and Chlorococcum (Brown and Bold, 1964, Brown and Lester, 1965). Others have used serological techniques to distinguish various species and mutants from each other (Brown and Walne, 1967; Gowans, 1963; Mintz and Lewin, 1954; Rosenblatt-Lichtenstein 1912, 1913; and Sanders, Keller and Wiley, 1971). Berns (1967) used the immunochemical characterization of biliproteins from various algae to set up a phylogenetic relationship among them. Codd and Schmid (1972) did the same type of investigation as Berns but by analyzing the glycolate-oxidizing enzymes

rather than pigments. Coleman (1963) and Hutner and Provasoli (1951, p. 116) tried to differentiate mating strains of 2 Pandorina species and 3 Chlamydomonas species respectively, but without success. In both of these genera, the mating gametes are morphologically similar and a distinct sperm and egg morphology is lacking. Since there is morphological sexual differentiation at the gamete level in Eudorina, possibly the male and female vegetative cells would be distinguishable immunologically.

MATERIALS AND METHODS

A. Organisms

Strains 1200 and 1201 of E. elegans were used as antigens. Strains 1192 and 1193 were also used in some of the agglutination tests. All strains were originally obtained from the Culture Collection of Algae at Indiana University (Starr, 1964).

B. Preparation of Antigens

Flagellar antigens, designated 1200f and 1201f, were prepared according to a modified method Coleman used to obtain flagella from Pandorina (Coleman, 1963). Coenobia from a 4-liter culture grown in BM were harvested by centrifugation at room temperature and rinsed once in sterile BM. Four volumes of a cold buffered 10% sucrose solution (see Appendix II) were added to 1 volume

of cells. The mixture was agitated for 2 to 3 min on a Vortex mixer, then left in an ice bath for 1 hr with occasional agitation. The mixture was centrifuged 7 min and the sediment containing the cells was discarded. The supernatant containing the flagella was then centrifuged 2 times for 4 min each to remove as many remaining cells as possible. The final supernatant was either temporarily frozen or else immediately ultracentrifuged at 20,000 x g for 20 min. The sedimented flagella were suspended in 0.15M NaCl buffered solution (see Appendix II) in approximately 1/10 of the supernatant volume.

Flagellar counts were made on a Petroff-Hausser bacteria counter using phase-contrast illumination. The final flagellar concentrations are listed in Table III-1 with an average of 2.96×10^7 flagella/ml.

Cellular antigens, designated 1200c and 1201c, were obtained from colonies grown on agar plates since no flagella were observed microscopically on cells grown on solid media. The antigens were prepared as acetone powder extracts using a method improved by Brown (1964). Colonies were grown on petri plates containing BC and 1.5% agar at 22°C for 2-3 weeks. Only plates uncontaminated by bacteria or fungi were used. The cultures were harvested by gently scraping the algae from the agar

Table III-1

Flagellar Concentrations After Centrifugation

Antigen	Ave. Concentration	No. of Readings
1200f	$2.80 \pm .58 \times 10^7$ fl/ml	5
1201f	$3.06 \pm .42 \times 10^7$ fl/ml	5
1193f	3.02×10^7 fl/ml	1

surface with the edge of a sterile microscope slide. The algal mass was then either frozen in a beaker for storage or directly transferred to a chilled mortar for immediate processing.

The mortar and pestle were chilled to about -15°C in an insulated bowl containing dry ice. The algal mass was added to the mortar, and after it froze solid, the algal pellet was gently crushed into a fine powder and enough acetone was added to form a very thick paste. Cell breakage occurred with considerable pressure on the pestle. The paste was also transferred to a glass tissue homogenizer in order to aid in cell breakage. Periodic microscopic checks for efficiency in cell breakage were made. When 75-80% of the cells had been broken, enough chilled acetone was added to form a slurry. This slurry was filtered at 4°C using a glass funnel and Whatman No. 1 filter paper. Two to 3 volumes of additional chilled acetone were added to further extract the fat-soluble material until a clear filtrate was obtained. The proteins and cellular debris, which are insoluble in acetone, were collected on the filter paper. The residue on the filter paper was dried at 4°C for 48 hrs. At room temperature the powder was scraped off the paper, placed in a mortar, and lightly

ground to a fine powder. The powder was weighed into 0.1 g aliquots and frozen until furthur use.

Powder suspensions, used for rabbit injections or absorption tests, were prepared by adding 2 ml of 0.15M NaCl buffered solution to 0.1 g of powder. The suspension was left at $4^{o}C$ for 2-24 hrs with occasional mixing in order to assure an homogenous mixture before it was used.

Protein content of the various antigens was determined using a micromethod of the Folin-phenol test (Lowry, Rosebrough, Farr and Randall, 1951). The protein determinations are shown in Table III-2.

C. Preparation of Antisera

Four young female rabbits were used for the production of antisera. Each rabbit received a different antigen -- 1200f, 1201f, 1200c, or 1201c. Table III-3 shows the schedule of injections and bleedings. On day 1, the rabbits were given 2 subcutaneous injections of 0.5 ml of the antigen thoroughly mixed with 0.5 ml of Freund's Complete Adjuvant. All other injections were done intraperitoneally using only the saline solution suspension. Control samples of blood were obtained by heart puncture on day 0 from the rabbits used as well as from 1 other rabbit. The rabbits were

Table III-2

Protein Concentrations of Antigens

of concentrated flagella and 0.1 g cells/2 ml.

Antigen	Protein Concentration
1200f	258 µg/ml
1201f	275 µg/ml
1193f	183 µg/ml
1200c	15.9 mg/ml
1201c	17.2 mg/ml

Table III-3

Schedule of Injections and Bleedings

Day	Injections to Rabbits with				Mode	Bleedings from each rabbit
	1200f	1201f	1200c	1201c		
0					HP	10 ml
1	2x.5 ml	.5 ml	2x.5 ml	2x.5 ml	SC	
8	.5 ml	.5 ml	.5 ml	.5 ml	IP	
15	.5 ml	.5 ml	.5 ml	.5 ml		
20					HP	10 ml
22	.5 ml	.5 ml	.5 ml	.5 ml	IP	
29	.5 ml	.5 ml	.5 ml	.5 ml	IP	
34	.5 ml	.5 ml	.5 ml	.5 ml	IP	
41					HP	30 ml
48					HP	30 ml
80					HP	30 ml
81	.5 ml	.5 ml	.5 ml	.5 ml	IP	
89					HP	30 ml

HP - heart puncture

IP - intraperitoneally

SC - subcutaneously

bled on 5 other occasions after injections began.

The blood was allowed to clot over a period of several hours and then centrifuged for 10 minutes. The serum was decanted into another tube and centrifuged again to remove any remaining cells. One drop of 2% sodium azide was added to every 4 ml of serum to insure sterility. The antiserum was then frozen until needed.

D. Immunological Tests

Agglutination tests were performed using antisera diluted to 1/40 and fresh algal cultures grown in BM of approximately 5×10^4 to 10^5 coenobia/ml. If complement was to be inactivated, the antiserum was heated to 60°C for 30 min. One drop each of serum and algal culture were placed in a spot plate and mixed by gentle oscillation. Any coenobial immobilization and flagellar agglutination was observed during the first 5 min using the low power lens of a compound microscope. The mixture was also checked at 30 and 60 min to insure the result was the same.

Sera that were absorbed were first incubated for 2 hrs at 22°C with cells from a fresh growing culture at a concentration of about 10^6 washed colonies to 1 ml of serum. This combination did not completely absorb the antibodies and agglutination still took place. The

ratio 2 x 10^6 colonies to 0.75 ml of serum was still not enough to accomplish complete absorption. However, if 5 x 10^6 colonies to 1 ml of serum were incubated for 1.5 hrs at 22°C and then the process repeated using new cells so there is a total of 3 hrs absorption, the antisera proved to be fully absorbed.

The Ouchterlony method (Ouchterlony, 1958) for double diffusion in gel was first used to test the precipitin reaction. Though diffusion was tested at 37°C, 22°C and 4°C, precipitin lines only appeared on agar plates at 4°C after 3-4 weeks. It was felt that an agar precipitin test, reported by Preer (1956), gave better and faster results.

For these diffusion tests, approximately equal volumes of antigen, agar, and antiserum were sequentially layered in glass tubes 4 cm long and 1.6-1.8 mm in diameter. The top was sealed with wax. The tubes were stored horizontally at 22°C and examined daily. Bands appeared within 2-3 d.

The agar used in the diffusion tests was 0.6% Difco purified agar (w/v) in 0.15M NaCl buffered solution. The agar solution was boiled 30 min, filtered with a Buchner funnel and Whatman No. 1 filter paper, and autoclaved. One ml of a 2% sodium azide solution

was added to each 100 ml of agar solution.

RESULTS

The results of the agglutination tests are presented in Table III-4. Two different phenomena were observed -- agglutination of the flagella and immobilization of the coenobia. No flagellar agglutination or coenobial immobilization took place if control serum or the buffered diluent were used as antisera. Inactivating the complement made no difference on the agglutination result. No difference between the 1200 and 1201 strains could be observed by immobilization or agglutination. A possible exception involved flagellar agglutination where 1200c and 1201c antisera did not cause agglutination as markedly with 1200 colonies, as with 1201 colonies.

One very interesting observation was made. When a male culture with sperm packets was used for the agglutination tests, the vegetative cells agglutinated and became immobile at the appropriate dilutions, but the sperm packets did not. At no time did the sperm packets immobilize or agglutinate. One can only conclude that the flagellar and cellular constituents of the vegetative cells, which were used as antigens, are different on the molecular level than those constituents present in the sperm.

Table III-4

Agglutination Tests

The results are the same whether or not the complement is inactivated.

ANTISERUM	Serum Dilution	ANTIGENS							
		1200 cells		1201 cells		1193 cells		1192 cells	
Control serum	1, ½, ¼, 1/8, 1/40	-	-	-	-	-	-	-	-
NaCl Buffered Diluent	1	-	-	-	-	-	-	-	-
1200f	1	++	xx	++	xx	+	x	+	xx
	½	++	xx	++	xx	-	-	-	xx
	¼	++	xx	++	xx	-	-	-	x
	1/8	+	xx	+	xx	-	-	-	-
	1/40	-	x	-	x	-	-	-	-
1201f	1	++	xx	++	xx	+	x	-	xx
	½	++	xx	++	xx	-	-	-	xx
	¼	++	xx	++	xx	-	-	-	xx
	1/8	+	xx	+	xx	-	-	-	-
	1/40	-	x	-	x	-	-	-	-
1200c	1	++	xx	++	xx	-	xx	-	xx
	½	++	xx	++	xx	-	x	-	xx
	¼	+	xx	++	xx	-	-	-	xx
	1/8	-	x	+	x	-	-	-	-
	1/40	-	-	-	-	-	-	-	-
1201c	1	++	xx	++	xx	-	x	-	xx
	½	++	xx	++	xx	-	-	-	xx
	¼	+	xx	++	xx	-	-	-	x
	1/8	-	x	+	xx	-	-	-	-
	1/40	-	-	+	x	-	-	-	-

++ flagella agglutination
+ some flagella agglutination
- no agglutination
xx immobilization
x some movement
- no immobilization

Using 1192 and 1193 strains to agglutinate with the antisera produced quite different results. The 1193 strain showed only partial immobilization in the undiluted serum. However, the 1192 Eudorina strain was immobilized in much the same way that 1200 and 1201 were, though not as strongly in the 1/8 dilution. Flagellar agglutination was questionable when undiluted 1200f antiserum was used. Otherwise, there was no flagellar agglutination.

There seems to be little difference between the flagellar and cellular antigens. Possibly this result is due to the similarity of the constituents, but more likely, it simply reflects the amount of cellular debris that contaminates the flagellar antigens and vice versa. Microscopic observations of the flagellar antigens revealed no cellular debris and cells grown on agar plates have no observable flagella. These observations, however, do not preclude that contaminating antigens are not present. Extremely small amounts of foreign antigens will induce antibody production.

Agglutination tests using completely absorbed antisera are shown in Table III-5. If antisera were absorbed using either 1200 or 1201 cells, no immobilization or flagellar agglutination took place with the undiluted

Table III-5

Agglutination Tests Using Absorbed Antisera

ANTISERUM	Absorbed with	Serum Dilution	ANTIGENS		
			1200 cells	1201 cells	1193 cells
Control serum	1200 cells	1	- -	- -	- -
1200f	"	1	- -	- -	- x
1201f	"	1	- -	- -	- x
1200c	"	1	- -	- -	- x
1201c	"	1	- -	- -	- x
1200f	1201 cells	1	- -	- -	- x
1201f	"	1	- -	- -	- x
1200c	"	1	- -	- -	- x
1201c	"	1	- -	- -	- x
1200f	1193 cells	1	++ xx	++ xx	- -
	"	$\frac{1}{2}$	++ xx	++ xx	- -
	"	$\frac{1}{4}$	++ xx	++ xx	- -
	"	1/8	+ x	+ x	- -
	"	1/40	- -	- -	- -
1201f	"	1	++ xx	++ xx	- -
	"	$\frac{1}{2}$	++ xx	++ xx	- -
	"	$\frac{1}{4}$	++ xx	++ xx	- -
	"	1/8	+ x	+ x	- -
	"	1/40	- -	- -	- -
1200c	"	1	++ xx	++ xx	- -
	"	$\frac{1}{2}$	++ xx	++ xx	- -
	"	$\frac{1}{4}$	+ xx	++ xx	- -
	"	1/8	- x	+ x	- -
	"	1/40	- -	- -	- -
1201c	"	1	++ xx	++ xx	- -
	"	$\frac{1}{2}$	++ xx	++ xx	- -
	"	$\frac{1}{4}$	+ xx	++ xx	- -
	"	1/8	- x	+ x	- -
	"	1/40	- -	- -	- -

++ flagellar agglutination
+ some flagellar agglutination
- no agglutination
xx immobilization
x some movement
- no immobilization

sera and either 1200 and 1201 cells. The reaction of 1193 cells with absorbed sera was not nearly as strong as with the unabsorbed sera. If 1193 cells were used to absorb antibodies in the sera, there was little difference in the results compared to unabsorbed sera except that the ability to immobilize 1193 cells was absent. Absorbed sera could not differentiate male and female cells by flagellar agglutination or coenobial immobilization.

The results are similar using Preer's diffusion techniques (Preer, 1956). Precipitation lines began to appear at 48 hrs and became most distinct by about 7 d as shown in Figure III-1. The line closest to the antigen appeared first and was most distinct using antisera 1200f and 1201f. It is presumed to be due to the flagellar antigens. One to three other lines appeared in 2 to 4 d and migrated towards the antiserum. These lines were strongest using 1200c and 1201c antigens and 1200c and 1201c antisera, so they are probably due to cellular antigens. The very slight differences in male and female antigens, such as 1200f and 1201f when diffused with antisera 1200c and 1201c are probably due to purity of the antigen or a slightly higher protein concentration in the female flagellar suspension. There were no precipitation lines with 1193 flagella or with control

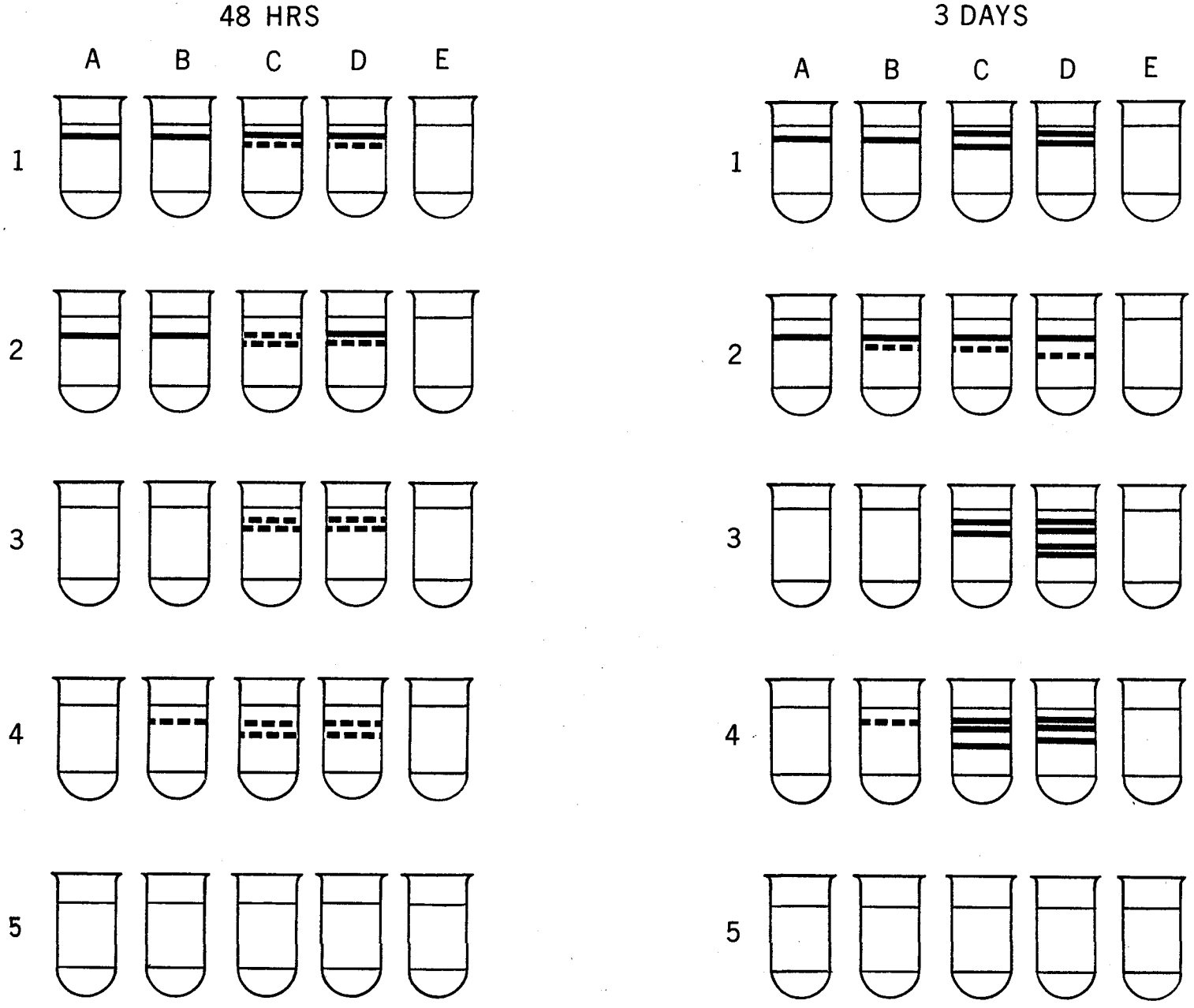
Figure III-1

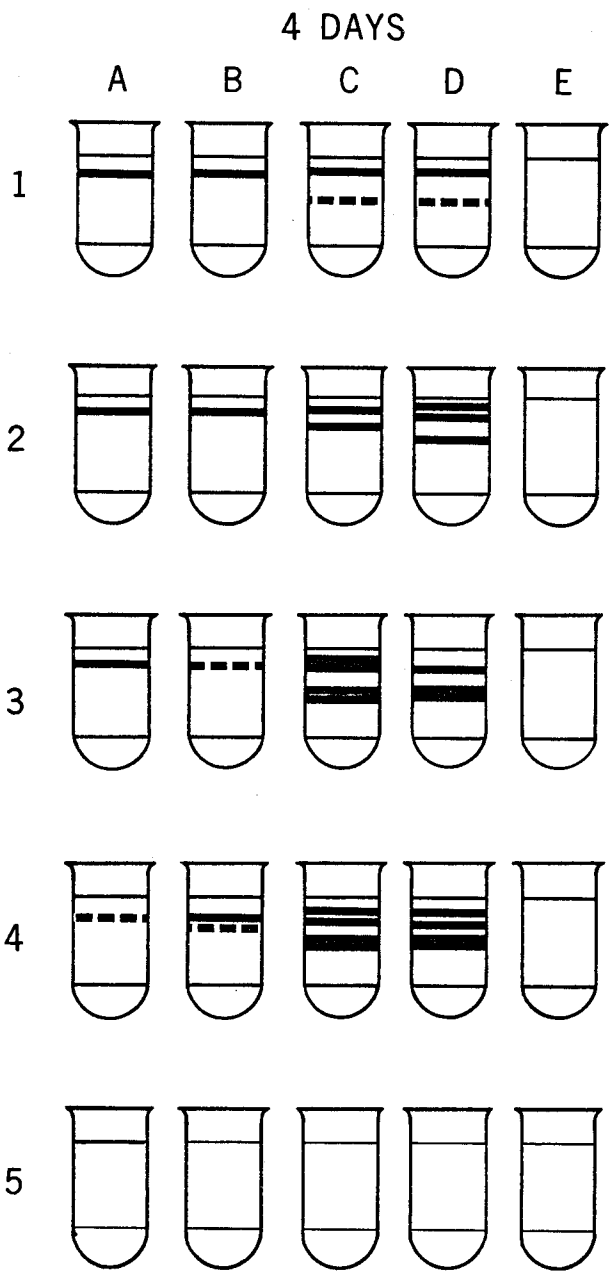
Diffusion Tests

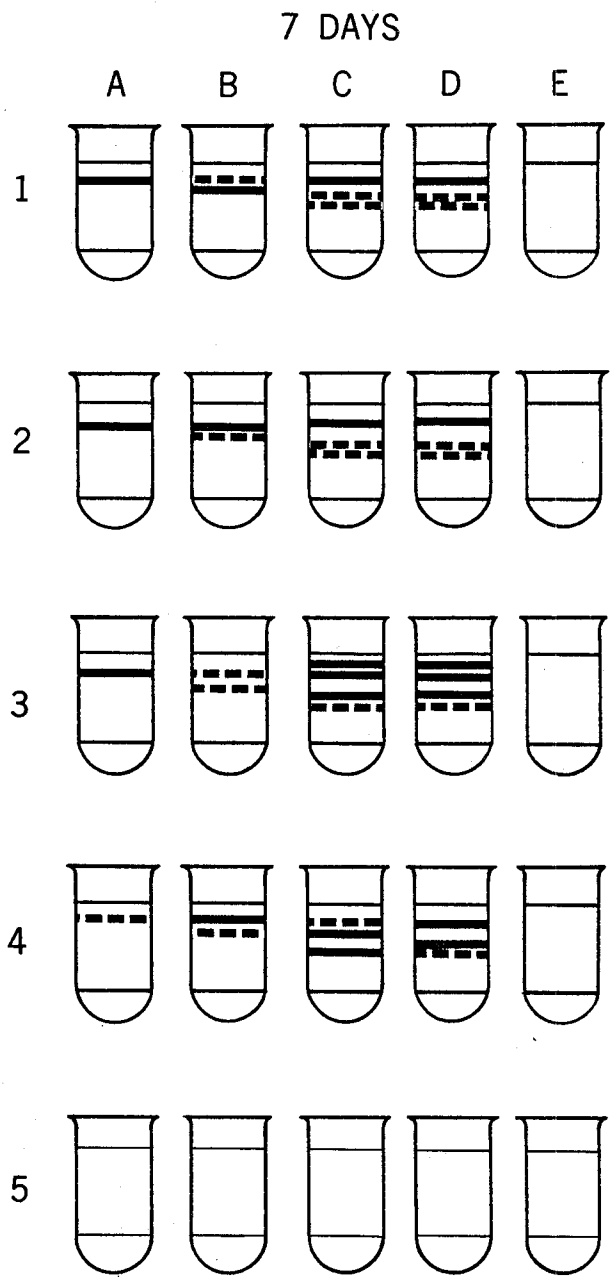
Equal volumes of antiserum, agar and antigen were sequentially layered in glass tubes (illustration is not to scale).

Agglutination lines were observed at 48 hrs, 3 d, 4 d and 7 d.

Antigens	Antisera
A - 1200f	1 - 1200f
B - 1201f	2 - 1201f
C - 1200c	3 - 1200c
D - 1201c	4 - 1201c
E - 1193f	5 - Control


48 HRS
A
B
C
D
E
1
2
3
4
5
3 DAYS
A
B
C
D
E
1
2
3
4
5


4 DAYS
A
B
C
D
E
1
2
3
4
5


7 DAYS
A
B
C
D
E
1
2
3
4
5

serum.

No differences among sera are detected if absorbed sera are used. If sera are absorbed with 1200 or 1201 cells, no precipitation lines occur up to 7 d. If 1193 cells are used for absorption as is shown in Figure III-2, the first and strongest line still appears using absorbed antisera from 1200f and 1201f while only questionable traces of the other lines are apparent. There appears to be no flagellar antibody absorption thus strengthening the conclusion that 1193 and 1200/1201 flagellar antigens are different. Some of the cellular antigens appear to be similar, since the cellular antigen lines hardly appear at all. However, distinct differences between the male and female antigens still could not be detected.

DISCUSSION

The result that 1200 and 1201 Eudorina strains could not be differentiated by immunological techniques merely confirms previous reports. Chlamydomonas mating types could not be differentiated with antisera prepared against cells or flagella (Hutner and Provasoli, 1951; and Mintz and Lewin, 1954). Antisera prepared against Pandorina flagella did not differentiate mating types either. In fact, the reactions even appeared to exhibit

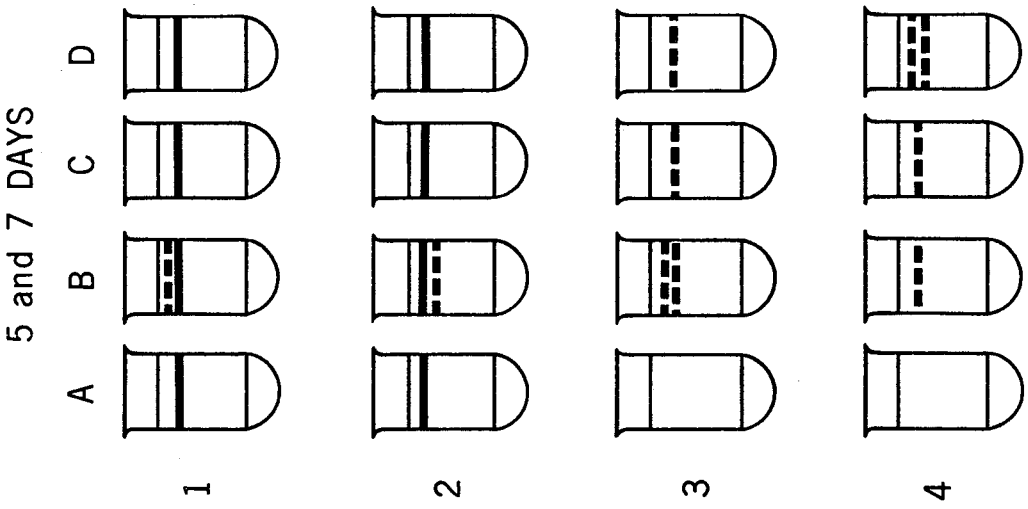
Figure III-2

Diffusion Tests Using Absorbed Antisera

Equal volumes of absorbed antiserum, agar and antigen were sequentially layered in glass tubes (Illustration is not to scale).

No agglutination lines were observed with sera absorbed with 1200 or 1201 cells. Lines were observed with sera absorbed with 1193 cells at 5 and 7 d.

Antigens	Antisera absorbed with 1193 cells
A - 1200f	1 - 1200f
B - 1201f	2 - 1201f
C - 1200c	3 - 1200c
D - 1201c	4 - 1201c


5 and 7 DAYS
A
B
C
D
1
2
3
4

syngen specificity because Pandorina strains that were isolated from different areas but were sexually compatible with each other, seemed to be similar antigenically (Coleman, 1963). The same result is found using E. elegans. The Eudorina strains 1192/1193 are in a different syngen than 1200/1201 (Goldstein, 1964). Antigenically, at least with respect to the flagella, the pairs are quite different as well.

One interesting aspect of the serological data is that the sperm do not agglutinate or immobilize with any of the antisera. There must be differences on the molecular level in both the flagella and cellular constituents. If there were a way of obtaining large amounts of male gametes separated from the vegetative cells, it would be of interest to carry out immunological tests to see just how different the male gametes are antigenically from the vegetative cells.

The fact that male and female Eudorina cells are antigenically similar supports the hypothesis that there is only one gene difference between mating strains in E. elegans (Goldstein, 1964). Other than the production of gametes and a possible hormone in the male, male and female E. elegans strains seem morphologically and biochemically identical. The genes coding for sperm

production and control of hormone production must be very close together, almost in tandem if not at the same locus, since these processes are exhibited in the male strain only.

It is unfortunate that male and female cells could not be differentiated by a simple agglutination test. It would certainly facilitate the genetic analysis of Eudorina. There are several other immunological procedures that could have been tried, such as electrophoretic techniques and fluorescent antibody staining. Problems probably would have been encountered with both procedures. The antigens seemed to diffuse very slowly through the agar-gel necessary for electrophoresis and cells containing chlorophyll fluoresce a bright red making fluorescent staining impractical. In either case, the purpose of this investigation -- to find an easy method of detecting male and female colonies -- would not be accomplished.

These immunological studies could be carried a good deal furthur. It would be interesting to see how different the mutants of 1200 and 1201 are from each other and the wild type strains. Results in Table III-4 suggests that the cellular antigens of 1192 are quite different than those of 1193. This difference is important considering 1192 and 1193 are mating strains according to

Starr (1964) but seem to have lost their sexual ability (Kemp, pers. comm.). The male strain 1192 does not produce sperm as it should. The difference in the immobilization reaction suggests that possibly a mutation(s) has occurred at the molecular level affecting the antigenic response as well as the compatibility with 1193 and the ability to produce sperm. Further immunological tests using 1192 and 1193 antigens might show exactly how different these strains are from each other now.

CHAPTER IV

GENETIC ANALYSIS OF EUDORINA ELEGANS

INTRODUCTION

Most genetic research in Phycology has been done with Chlamydomonas reinhardi (reviewed in Levine and Ebersold, 1961). Studies involving other members of the order Volvocales have shown a 1:1 segregation of mating type alleles indicating the mating type is under the control of a single pair of alleles (Gonium pectorale, Stein, 1958; Pandorina morum, Coleman, 1959; Eudorina elegans and Eudorina illinoisensis, Goldstein, 1964; Volvulina steinii, Carefoot, 1966; and Astrephomene gubernaculifera, Brooks, 1966). Besides Goldstein's investigations on Eudorina, Mishra isolated several drug resistant mutants in E. elegans -- namely mutants resistant to sulfoximine, streptomycin, or erythromycin (Mishra and Threlkeld, 1968). The streptomycin-resistant marker was found to be inherited cytoplasmically through the female parent while the other two markers were inherited in a Mendelian fashion. None of these markers were linked to the mating type locus.

Kemp (unpubl.) has isolated a number of chlorophyll deficient mutants of Eudorina elegans in the 1200 and 1201 strains. He also has a few morphological mutants such

as granular, single celled, and clumpy colonial forms. Only one auxotrophic mutant requiring nicotinamide has been isolated in 1201.

The main problem was to mate these mutants with the wild type strain and with each other, analyze the ratios of progeny, and attempt to construct a genetic map showing any linkage groups found.

If any of the mutant phenotypes are uniparentally inherited, the phenotype of the female parent would be expressed in the progeny (Mishra and Threlkeld, 1968) as is diagrammed in Figure IV-1, crosses a and b. If any of these mutations are nuclear in origin, a 1:1 ratio of mutant and wild type classes should appear in the F_1 generation. The results of independent assortment or linkage between two markers are shown in Figure IV-1, crosses c and d respectively. Any significant deviation from a 1:1:1:1 ratio of the possible phenotypes indicates linkage between the two markers. Since only one gone cell emerges from the zygote, tetrad analysis is not possible as in Chlamydomonas where there are usually 4 or 8 meiotic products.

MATERIALS AND METHODS

A. Organisms

The mutants used in this analysis were all derived

Figure IV-1

Diagrams of Inheritance Patterns

If Uniparental Inheritance:

Cross a)

P_1 1200 x 1201-m
phenotype wildtype x mutant
F_1 1 1200-m : 1 1201-m
phenotype all mutant

Cross b)

P_1 1200-m x 1201
phenotype mutant x wildtype
F_1 1 1200 : 1 1201
phenotype all wildtype

If Mendelian Inheritance:

Cross c)

mutant (m) assorts independently to sex alleles

P_1 1200 x 1201-m
phenotype : wildtype x mutant
F_1 1 1200 : 1 1200-m : 1 1201 : 1 1201-m
phenotype wildtype : mutant : wildtype : mutant

Cross c)

mutant (m) is linked to sex alleles

P_1 1200 x 1201-m
phenotype wildtype x mutant
F_1 1 1200 : 1 1201-m : very few 1200-m : very few 1201
phenotype wildtype : mutant ; mutant wildtype
parental types recombinant types

by Kemp from the 1201 strain of Eudorina elegans using UV irradiation as the mutagen. These mutants are described in Table IV-1 and are shown in Figures IV-2, 3 and 4. Other mutants have been isolated in the 1200 and 1201 strains, but these did not appear to be sexually competent and were not analyzed.

B. Procedure

The procedure for zygote formation and germination is similar to that outlined in Chapter II but with more emphasis in keeping the zygotes sterile. Male and female cultures from frequently transferred liquid stocks were grown separately for 1-3 d in VM at 32°C. Three ml of each mating type were placed in a sterile flask or test tube and incubated at 32°C with a 16:8 cycle for 8-10 d. The zygotes were then spun down and the supernatant discarded. Zygotes were stored in test tubes at room temperature in darkness for 1 week. The test tubes with the dried zygotes were placed in a 37°-40°C oven for 3-5 d in order to induce germination. BC was added to the test tubes which were then placed in the light at 32°C. After 24 hrs the medium was replaced with fresh BC. Twenty-four hours later, the zygotes started to germinate and they were immediately plated on BC 1.5% agar plates with 0.6% top agar. The

Table IV-1

Description of Mutants Used in Genetic Analysis

Number	Description
1200	Wildtype male
1201	Wildtype female
1201-8	Clumpy, irregularly large colonial form, small dark colony on plate
1201-40	Clumpy, irregularly large colonial form, small dark colony on plate
1201-13	Dark, large dark colonial form in liquid, small dark dry colony on plate
1201-21	Granular, large colonial form in liquid, mucoid granular-looking (with distinct coenobia) colony on plate
1201-2	light green in liquid and on plate
1201-3	light green in liquid and on plate
1201-7	light green in liquid and on plate
1201-46	light green in liquid and on plate
1201-22A	light green in liquid and on plate
1201-38	light green in liquid and on plate
1201-11	light green - a bit lighter than above 6 mutants
1201-32	pale green in liquid and on plate
1201-44	very pale green - paler than 1201-32
1201-9	very pale green - paler than 1201-32
1201-61	requires nicotinamide (approximately 1 µg/ml) for growth

Figure IV-2

Mutants of E. elegans 1200/1201

Mutants -2, -3, -7, -8, -9 and wildtype are shown.

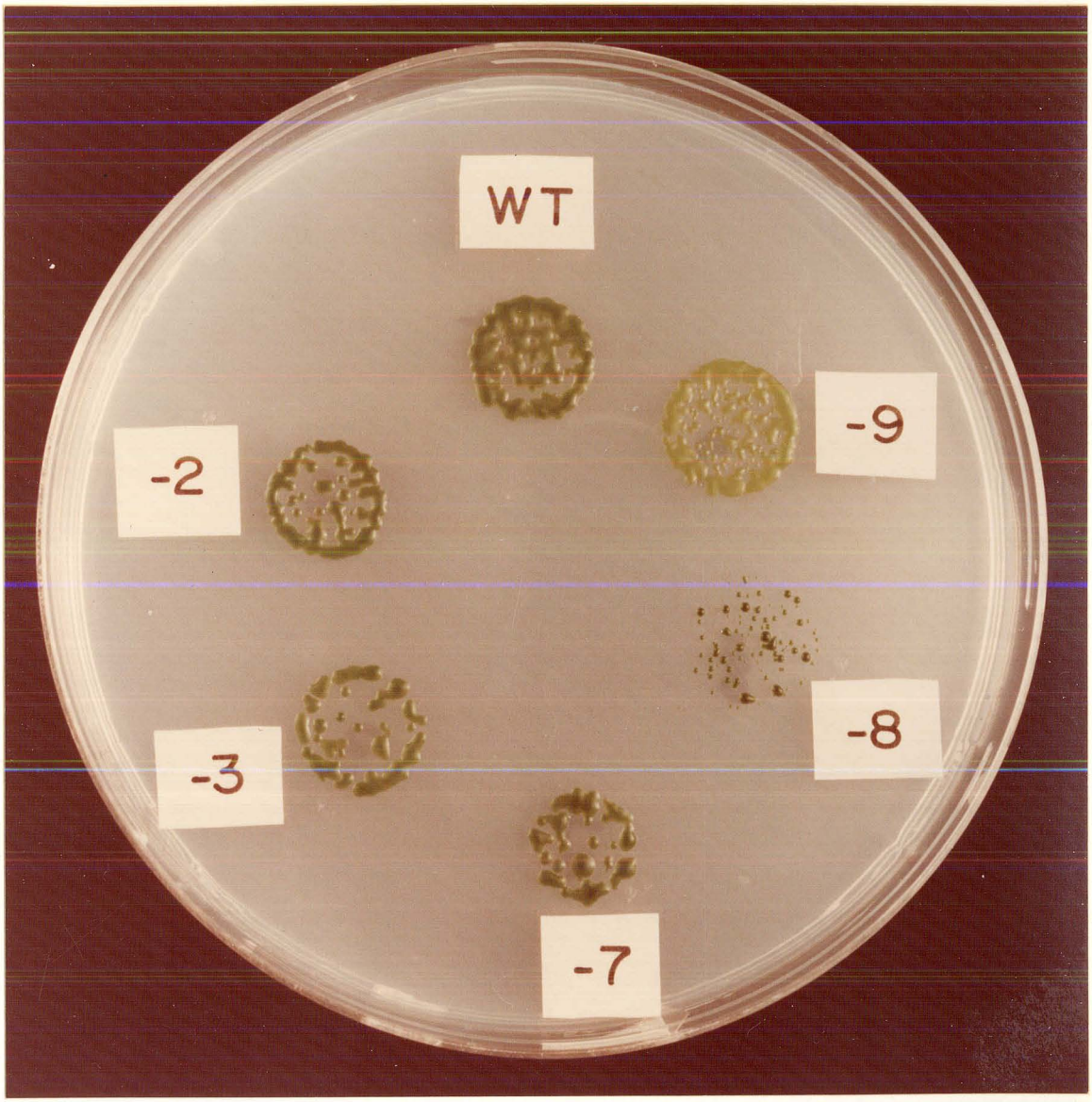


Figure IV-3

Mutants of E. elegans 1200/1201

Mutants -11, -13, -21, -22A, -32 and wildtype are shown.

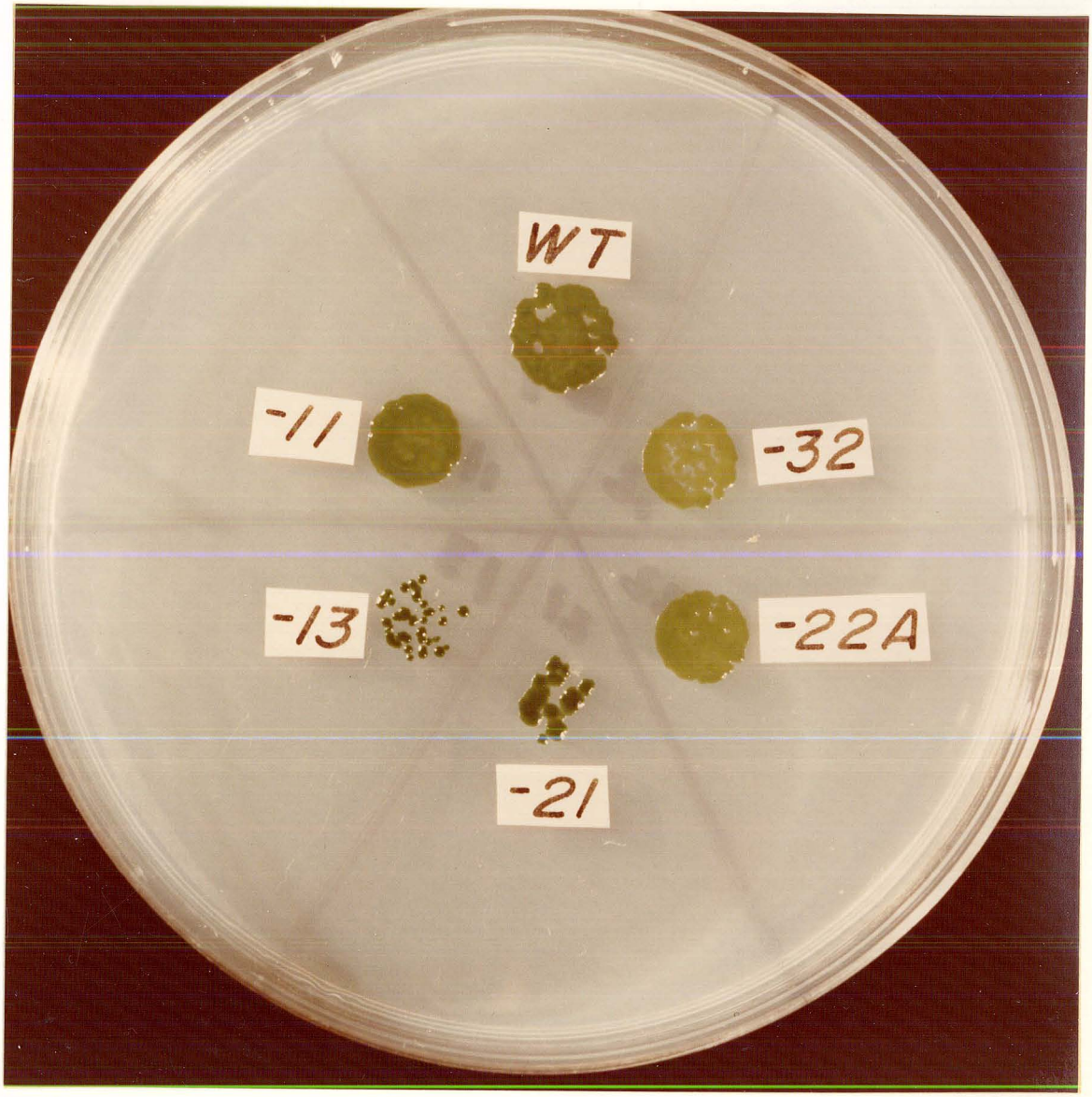
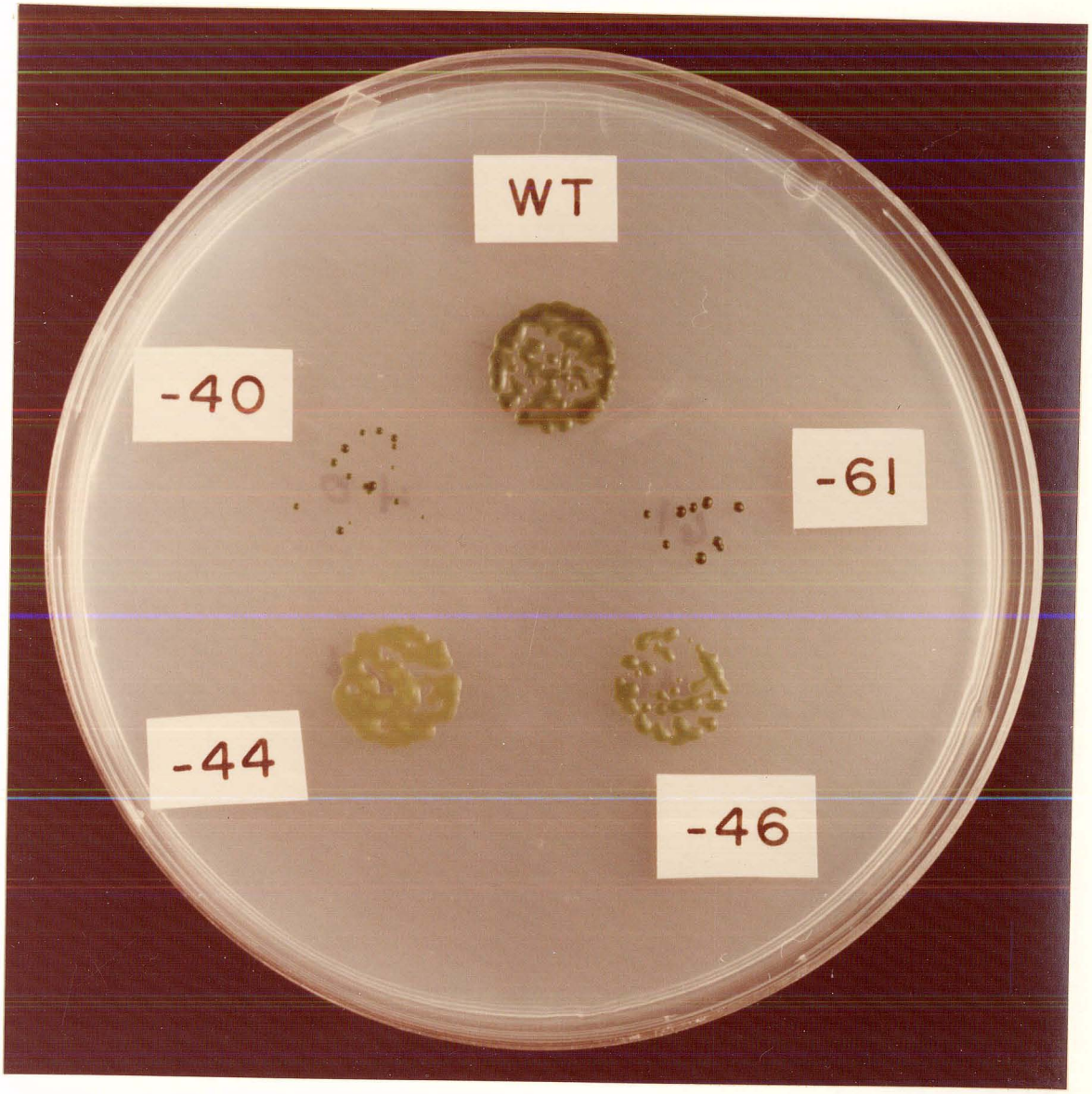


Figure IV-4

Mutants of E. elegans 1200/1201

Mutants -40, -44, -46, -61 and wildtype are shown.



plates were incubated about 10 d at 32°C.

C. Mutant and Mating Type Identification

There were a few problems with the identification of the markers used in this investigation. Color differences were most distinct if the colonies on the plates were 10-14 d old. The pale mutants, -9 and -44 are not distinguishable from each other. The same is true of the light green mutants. However, the pale mutants are distinctive from the light green ones. Mutant -32 is in between the pales and the light greens in color and cannot be distinguished from pale or light green colonies when growing on the same plate.

Gone colonies were picked from the plates and grown in liquid medium for male-female analysis. The colonies were inoculated into BC, grown for several days, and then transferred into VM for sexual induction. After 3 d those cultures producing sperm clumps were classed as males. The other cultures were crossed with known female and male strains in watch glasses or spot plates. The sex is identified by the ability of the isolate to produce zygotes with the opposite sex.

To see if the gone colonies from crosses with -61 required nicotinamide, or not, the colonies were inoculated into BC, grown for several days, washed twice with

phosphate buffer, and then transferred onto BM and BC plates. Wild types will grow well on both plates. Nicotinamide-requiring mutants will grow well on BC plates but will bleach white and die on the BM plates.

Progeny from clumpy mutants (-8, -40) crosses were grown in liquid to assure their growth was normal or clumpy, though clumpy mutants could usually be distinguished from the wild types on plates by their dark dry small colonial form.

A dissecting microscope was used to distinguish the granular-looking, mucoid morphology of -21 colonies from the smooth wild type form.

D. Analysis of Data

Chi square was used to analyze deviations from expected ratios obtained in the various crosses. If the deviation from an expected independent assortment was significant, recombinational distance was then calculated in 2 ways. Where the double mutant is present and phenotypically distinct from the single mutants, such as in the cross 1200-11 x 1201-21, the recombinational distance (d) is:

$$d = \frac{w + m}{T} \times 100\%$$

where w = number of wild type recombinants
m = number of double mutant recombinants
T = total number of progeny

If the double mutant is not distinguishable from the single mutant, such as in the cross 1200-9 x 1201-44, the recombinational distance is expressed in a range from d_1 to d_2 -- the distance being d_1 if the double mutant were present but phenotypically similar to the single mutants, the distance being d_2 if the double mutant were lethal.

$$d_1 = \frac{2 \times w}{T} \times 100\%$$

$$d_2 = \frac{2 \times w}{T + w} \times 100\%$$

where w = number of wild type recombinants
T = total number of progeny

Both calculations are made since either outcome is possible. A selective disadvantage is calculated for some of the mutants where the deviation from the expected ratio in a mutant-wild type cross is significant. In this case, the selective disadvantage (s) is:

$$s = 1 - \frac{o}{e}$$

where o = observed number of mutants
e = expected number of mutants

RESULTS

Crosses were attempted with a number of mutants without success. Only those mutants that could be distinguished from the wild type and were sexually inducible were used for genetic analysis. The stability

of some of these mutants is still in question, although control plating of the vegetative cells at the onset of the series of crosses showed no wild type revertants. If 1201-40 is kept in liquid medium for a long period of time, wild type revertants will eventually overgrow the clumpy, slower growing mutants. Table IV-2 shows the results when the mutant strains are crossed with themselves (the males being derived from wild type-mutant crosses which are outlined in Table IV-3) and the phenotypes of the progeny. Though some numbers of total progeny are too low to observe any revertants even if they did exist, wild type revertants were obtained in 2 crosses 1200-44 x 1201-44 and 1200-46 x 1201-46. The percent of wild type progeny, .414% and 3.65% respectively, is high if considered as the amount of reverse mutations.

Because most of the mutants used are color mutants, it is of interest to see whether these mutations are cytoplasmic or nuclear in origin. Table IV-3 shows the results from the wild type-mutant crosses. With the exception of 1201-38, all of the mutants are inherited in a Mendelian fashion giving a 1:1 ratio of mutants to wild types. This 1:1 segregation also supports the view that these mutants are derived from single, and not double, mutations. The crosses with the clumpy

Table IV-2

Mutants Crossed with Selves

Cross	Total # of Progeny	Phenotype of Progeny	Wildtype %
1200-2 x 1201-2	6	all light green	-
1200-3 x 1201-3	75	all light green	-
1200-7 x 1201-7	47	all light green	-
1200-8 x 1201-8	-	-	-
1200-9 x 1201-9	61	all pale	-
1200-11 x 1201-11	>3,000	all light green	-
1200-13 x 1201-13	155	all small, dark	-
1200-21 x 1201-21	>3,500	all granular	-
1200-22A x 1201-22A	227	all light green	-
1200-32 x 1201-32	-	-	-
1200-44 x 1201-44	725	722 pale, 3 wildtype	0.414%
1200-46 x 1201-46	438	422 light green, 16 wildtype	3.65%
1200-61 x 1201-61	-	-	-

Table IV-3

Wildtype-Mutant Crosses

Cross	Progeny		Probability of deviation from 1:1 ratio	Selective disadvantage of mutant
	Mutant	Wildtype		
1200 x 1201-2	306	280	p>.05	
1200 x 1201-3	239	232	p>.05	
1200 x 1201-7	93	99	p>.05	
1200 x 1201-8	78	123	p<.01*	s = .366
1200 x 1201-9	293	257	p>.05	
1200 x 1201-11	331	354	p>.05	
1200 x 1201-13	52	67	p>.05	
1200 x 1201-21	121	128	p>.05	
1200 x 1201-22A	190	205	p>.05	
1200 x 1201-32	189	173	p>.05	
1200 x 1201-38	-	53	p<.01*	s = 1
1200 x 1201-40	5	17	.01<p<.05**	s = .706
1200 x 1201-44	463	433	p>.05	
1200 x 1201-46	95	81	p>.05	
1200 x 1201-61	38	33	p>.05	

* deviation significant
** deviation questionable.

mutants give significantly fewer mutant progeny than expected. A selective disadvantage has been calculated for these mutants. An unexpected result was obtained with 1201-38, a light green mutant, when only wild type and no mutant offspring were obtained.

In order to find out if any of the mutants are linked with the sex alleles, usually 40 progeny of both wild type and mutant phenotypes were analyzed for male-female ratios. There appearsto be a 1:1 segregation of males to females in all groups as shown in Table IV-4. The only questionable exception is the mutant offspring from the 1200 x 1201-21 cross. In that instance the other phenotypic class, the wild type progeny, does show 1:1 segregation. Considering the total number of progeny involved, the one significant deviation is probably not that important and simply due to chance. There does seem to be independent assortment of these markers with respect to the mating type alleles. In the cross 1200 x 1201-40, no sperm producing males were obtained with a clumpy phenotype while sperm packets produced by the mutant males from 1200 x 1201-8 cross were definitely abnormal in their morphology and swimming ability.

The mutant-mutant crosses were attempted at least 6 times but they were not always successful. Even if

Table IV-4

Sex Ratios from Wildtype-Mutant Crosses

Cross	F_1 phenotype	Total F_1 Offspring Used	Females	Males	Colonies died or not differentiated	Probability of deviation from 1:1 ratio
1200 x 1201	wildtype	144	70	72	2	p>.05
1200 x 1201-2	light green	40	14	26	0	p>.05
	wildtype	40	25	15	0	p>.05
1200 x 1201-3	light green	40	21	19	0	p>.05
	wildtype	40	22	18	0	p>.05
1200 x 1201-7	light green	40	16	24	0	p>.05
	wildtype	40	22	15	3	p>.05
1200 x 1201-8	clumpy	48	14	10	14	p>.05
	wildtype	48	28	19	1	p>.05
1200 x 1201-9	pale	40	17	11	12	p>.05
	wildtype	40	21	19	0	p>.05
1200 x 1201-11	light green	40	23	17	0	p>.05
	wildtype	40	23	17	0	p>.05
1200 x 1201-13	small dark	40	26	14	0	p>.05
	wildtype	40	21	19	0	p>.05
1200 x 1201-21	granular	48	33	15	0	p=.01*
	wildtype	48	25	22	1	p>.05
1200 x 1201-22A	light green	48	26	22	0	p>.05
	wildtype	48	27	21	0	p>.05
1200 x 1201-32	pale	40	20	17	3	p>.05
	wildtype	40	18	21	1	p>.05
1200 x 1201-38	wildtype	20	9	11	0	p>.05
1200 x 1201-40	clumpy	5	3	0	2	-----**
	wildtype	17	7	10	0	p>.05
1200 x 1201-44	pale	40	15	25	0	p>.05
	wildtype	40	23	15	2	p>.05
1200 x 1201-46	light green	40	22	16	2	p>.05
	wildtype	40	19	21	0	p>.05
1200 x 1201-61	nicotinamide-	38	10	11	17	p>.05
	wildtype	33	14	19	0	p>.05

* deviation significant

** chi not calculated.

zygotes were obtained, they did not always germinate or the per cent germination was sometimes extremely low. The mutant-mutant crosses producing offspring are listed in Appendix III complete with chi square values and recombinational distances. Table IV-5 summarizes the crosses showing when successful zygote formation and germination occurred. As can be seen, some mutants seem more sexually viable than others, namely -11, -9, -21, -22A, and -44. Those mutants that seem quite incompatible with the other mutants are -8, -32 (especially 1200-32), -40, and -61. Only rarely were zygotes even obtained in wild type-mutant crosses using these mutants. Those showing partial incompatibility with only certain mutants are -3 with the pale mutants; -7 with the pales, -2, and -3; and -13 with -2 and -3 as well.

All of the crosses listed in Appendix III were analyzed using chi square, if the total number of progeny were over 50. If the deviation from an expected independent assortment was not significant, the markers used were considered not to be linked. If the probability of the deviation was less than .05, the recombinational distance was calculated. Table IV-6 summarizes the results of the mutants linked together or assorting

TABLE IV–5
MUTANT–MUTANT CROSSES

	−8	−40	−13	−21	−61	−2	−3	−7	−46	−22A	−11	−32	−44	−9
−8														
−40														
−13			Z G											
−21	Z –		ZZ GG	Z G										
−61														
−2				ZZ Gg		Z g								
−3				ZZ Gg		Z G	Z g							
−7			Z G	Z G			Z g	Z g						
−46			ZZ GG	ZZ GG		Z G	ZZ g–	Z G	Z G					
−22A			ZZ GG	ZZ GG		ZZ GG	ZZ gG	Z G	ZZ GG	Z G				
−11	Z g		Z G	Z G	Z g	ZZ GG	ZZ GG	Z G	ZZ GG	ZZ GG	Z G			
−32						Z G	Z g	Z g	Z –	ZZ Gg				
−44			Z G	ZZ gG	Z g	ZZ G–	Z –	ZZ gG	ZZ GG	ZZ Gg	ZZ GG	Z G	Z G	
−9	Z g	Z g	Z G	Z G		Z G		ZZ GG	ZZ GG	ZZ gg	ZZ gG	Z G	ZZ GG	Z G

Z Zygotes from one cross
ZZ Zygotes from reciprocal crosses
g Less than 50 gone colonies
G More than 50 gone colonies
– No germination

TABLE IV–6

LINKAGE DISTANCE BETWEEN MUTANTS
(based on recombinational data in Appendix III)

	−8	−40	−13	−21	−61	−2	−3	−7	−46	−22A	−11	−32	−44	−9
−8														
−40														
−13														
−21			13.0* -12.2											
−61														
−2				NL*										
−3				NL*		39.0 -32.6								
−7			NL	NL			NL							
−46			NL*	37.0*		38.8 -32.5	NL	NL						
−22A			18.4*	NL*		NL*	NL*	NL	42.8* -35.3					
−11	NL		45.0 -36.8	15.5		33.6* -28.8	NL*	NL	NL*	NL*				
−32						19.6 -17.9		NL		31.3* -24.8				
−44			NL	NL*	NL	33.3 -28.5		11.5* -10.8	20.3* -18.4	NL*	34.8* -29.7	NL		
−9	NL		NL	NL		23.0 -20.6		16.9* -15.6	18.1* -16.7	NL*	19.6* -17.9	NL	6.1* 5.9	

NL = not linked

* = data from reciprocal crosses.

range of linkage distance due to questionable viability of double mutant recombinant

independently. The recombinational distance in the table is calculated from the sum of progeny from reciprocal crosses when both crosses were successful.

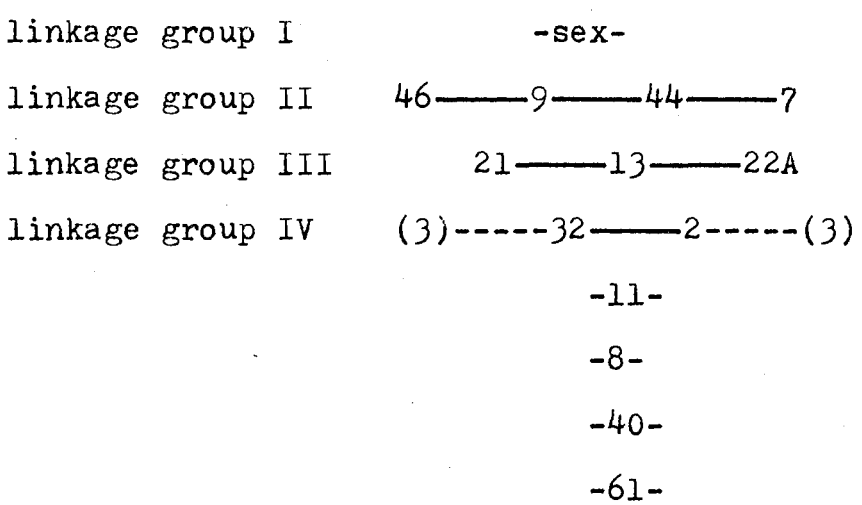
In construction of a linkage map using the distances obtained from the crosses, discrepencies do arise. The markers -46, -9, -44, and -7 show good linkage. The mutants -21, -13, and -22A seem to be linked together. Mutant -2 shows linkage with -3 and -32. But the position of the group -3, -2, and -32 and the position of mutant -11 is confusing since they show linkage to mutants in both of the other groups. The position of mutants -8, -40, and -61 are not known because of their incompatibility with the other mutants. Since wild type offspring were obtained in crosses 1200-11 x 1201-8, 1200-9 x 1201-8, and 1200-44 x 1201-61, mutant -8 is probably not linked to -11 and -9, and mutant -61 is probably not linked to -44. Figure IV-5 summarizes the groups with no speculation as to how the groups and unplaced mutants are linked together.

DISCUSSION

None of the mutants studied show uniparental inheritance through the female when crossing them with the wild type strain. Even the chlorophyll deficient mutants are under nuclear, rather than cytoplasmic,

Figure IV-5

Summary of Linkage Groups



control. Mishra (1967) found that streptomycin-resistant mutants did show uniparental inheritance in Eudorina, so a system of uniparental inheritance appears to exist for this alga. Most of the non-chromosomal genes demonstrated in Chlamydomonas reinhardi are associated with antibiotics that directly affect organelles like the mitochondria or the chloroplast (Sager and Ramanis, 1970). Mutations that affect chloroplast development or chlorophyll synthesis in Chlamydomonas reinhardi have shown Mendelian inheritance (Surzycki, Goodenough, Levine and Armstrong, 1970). These chlorophyll deficient mutants in E. elegans are no exception; they, too, show Mendelian inheritance.

When crossing these mutants with the wild type, a 1:1 segregation resulted. This ratio shows that these mutants are due to single mutations. If one had been due to 2 mutations, a very rare event, where each mutation gave expression to the phenotype, a 3:1 ratio would have resulted.

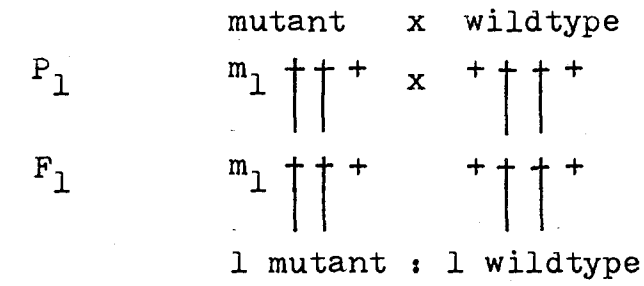
The idea of a redundant genome might cause a few problems in genetic analysis. Chromosome numbers do vary among species in the same genus (reviewed in Godward, 1966, pp. 10-14). Chlamydomonas species contain 8-38 chromosomes; Volvox species can have from 5 to 15 chromosomes; and E. elegans has been reported to have from

10 to 16 chromosomes. So it is feasible that some chromosomes are duplicates in Eudorina. If a mutation is present in only one of a pair of similar chromosomes in a vegetative cell and is expressed, it would be considered dominant over the wild type allele. If redundancy is the case, there is still a 1:1 segregation in wild type-mutant crosses as illustrated in Figure IV-6. In mutant-mutant crosses whose single mutant loci are on homologous chromosomes, but chromosomes that are duplicated, more wild type progeny, than expected due to independent assortment, may arise due to random pairing and segregation of these chromosomes in the zygote state. If the mutant loci are closely linked, the number of wild type offspring will approach 25%. If the mutant loci are 50 map units apart, the number of wild types will approach 37.5%. Several of the mutant-mutant crosses do show the percent of wild type progeny within this range. However, there is no group of mutants all showing this pattern. Most of these mutant-mutant crosses are with mutant -13 which also show fewer small dark colonies than expected. A more probable explanation, rather than redundancy, for the increased number of wild types is that the -13 culture had wild type revertants which mated in the mating

Figure IV-6

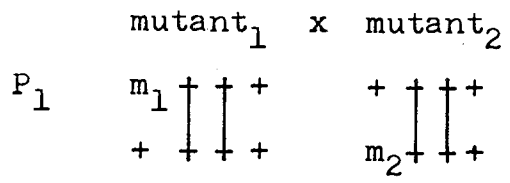
Theoretical Results with Duplicate Chromosomes Present in Gametes

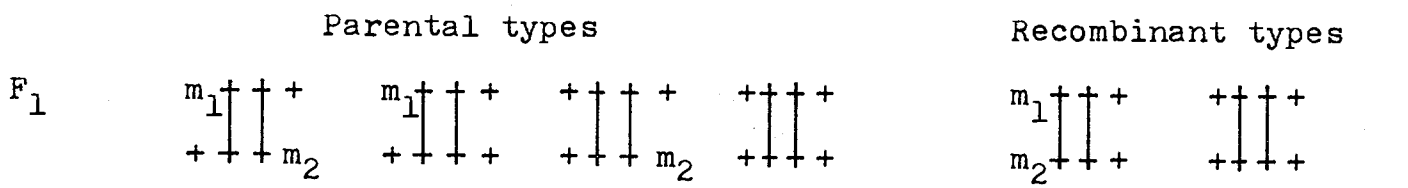
A. Mutant-wildtype Cross



1 mutant : 1 wildtype

B. Mutant-Mutant Cross





if d = 0	25%	25%	25%	25%	0	0
if d = 50	12.5%	12.5%	12.5%	12.5%	25%	25%

if d= 0, total wildtype = 25%

if d = 50, total wildtype = 37.5%

dish as well.

The stability of some of these mutants besides -13 is still in question. Control platings of the vegetative cells at the onset of the series of crosses showed no wild type colonies present. However, the percent of wild type progeny, .414% and 3.66%, obtained in crosses 1200-44 x 1201-44 and 1200-46 x 1201-46 respectively is extremely high if considered as the amount of reverse mutations. Since both strains are grown in liquid medium before mating, there is a good possibility that the reversion occurred, not in the zygote, but in the vegetative cell state which then could replicate, participate in zygote formation, and show itself in the progeny. Though no true fraction of reverse mutations has been calculated, the possibility that some wild type progeny in mutant-mutant crosses could be due to reversions and not recombinations should be considered.

Because very little is known about the physiology of the mutants used in this genetic analysis, there are possibilities of errors simply due to the mutants themselves. Germination was very low, usually less than 20%. Some of the mutants, such as -61 or -8, gave very little germination, if any at all. The possibility of the mutant being lethal in the zygote state is quite

feasible. A selective disadvantage has been calculated for -8 and -40 since there were significantly fewer mutant than wild type progeny. Sporadic germination as well as the physiological state of the mutants in either the zygote or gone cell could cause variation in the ratios of F_1 phenotypes.

The results obtained with -38, when crossed to the wild type, are strange. Since a 1:1 segregation is not obtained, nuclear control of the light green phenotype is not probable. But if it were under cytoplasmic control, uniparental inheritance is expected through the female parent. However, only the wild type phenotype of the male parent is expressed in the progeny. Uniparental inheritance through the male is not out of the question, but it is unprecedented. Mishra (1967) has already observed uniparental inheritance through the female in Eudorina. There is the possibility that meiosis did not occur and the offspring are diploid expressing the dominant wild type phenotype. But the offspring consisted of both males and females, so the progeny are products of meiosis where nuclear segregation did occur. Possibly the -38 mutation is lethal in the post meiotic zygote or gone cell state. There is also the possibility that zygotes were formed only with wild type revertants present in the liquid culture of

-38. The phenotype might even be due to some instability in the cell that is not genetic in origin and is not inheritable. Unfortunately, 1201-38 was lost due to contamination and it was not possible to cross it with any of the mutants to see if the phenotype was stable and inheritable.

None of the markers used in this analysis are linked to the mating type locus. The mutant-wild type crosses showed a 1:1 ratio of males to females in each phenotypic group. The drug-resistant markers showing Mendelian inheritance that Mishra used demonstrated independent assortment with the mating type locus in Eudorina as well (Mishra, 1968). Two nicotinic mutants show very close linkage to the mating type locus in Chlamydomonas reinhardi (Ebersold, Levine, Levine, and Olmsted, 1962). It would be very useful in future genetic analysis if a closely linked marker to the sex alleles could be found in Eudorina. Then sexual differentiation would merely consist of scoring the appropriate phenotypic colonies on agar plates.

There are some interesting observations of the results from the mutant-mutant crosses concerning which mutant is linked to which. Mutants -9 and -44 are both pale mutants which are not distinguishable on

a plate. The loci are linked with 5.9 to 6.1 map units between them. Mutants -13 and -21 are microscopically similar showing very distinct large dark cells in each coenobium, though on a plate -13 has much smaller and dryer colonies than -21 whose colonies are more mucoid and granular-looking. These 2 mutants seem to be linked together by 13.0 to 12.2 map units. It is also of interest that wild type recombinants were obtained only from the 1200-21 x 1201-13 cross and none at all were obtained from the reciprocal 1200-13 x 1201-21 cross. The origin of the wild type offspring from the one cross might be in question which would link these 2 mutants even closer together. Possibly the locus on the chromosome responsible for paleness versus wild type, or for the size and shape of the vegetative cells, is quite large and different mutations can be induced within this area that shows linkage and gives similar phenotypes. However, the large map distances between the similar markers does decrease the possibility of a polygenic locus. The light green mutants, which are more prevalent and easier to obtain by mutagenesis, show no allelism. The light green phenotype can possibly be due to various blocks in metabolic pathways which are regulated by a number of loci and not just one particular

area on a chromosome. Chlorophyll deficient mutants in Chlamydomonas have also been mapped in a number of linkage groups and not just one locus (Surzycki, et al., 1970).

Some of the mutants are not very competent sexually while others are very sexually viable. Because of the morphology of the clumpy mutants, -8 and -40, and the irregular-looking sperm packets of 1200-8, it is conceivable that their incompatibility with the other mutants and wild types are simply due to their morphology. However, 1200-32 and 1200-61 both can produce large numbers of healthy-looking sperm and neither has been able to produce zygotes with the other mutants. Nutritional requirements might be the reason for sexual inviability, as Stein has shown is the case in some intra-species crosses with Gonium pectorale (Stein, 1966). Other combinations, such as -3 crossed with the pale mutants and -7 or -13 crossed with -2 and -3, might also require other nutrients not present in VM or synthesized by either of these mutants. Immunological studies of these mutants might show antigenic differences which could be correlated with their incompatibility. Possibly because of molecular changes, these mutants are so different structurally that fertilization with each other or zygote

germination is not possible. This reasoning, however, does not explain why no zygotes are formed in the 1200-61 x 1201-61 and 1200-32 x 1201-32 crosses. A deficiency in necessary nutrients might be the case for at least these two mutants.

The actual number of chromosomes in E. elegans 1200 and 1201 strains is still uncertain. Goldstein did not report a chromosome number for these strains (Goldstein, 1964) and photographs made by Ken Lee (pers. comm) in our laboratory show extremely small chromosomes that are almost impossible to count accurately. The range of 10 to 16 chromosomes for E. elegans counted by various researchers (Godward, 1966, p. 11) has not been improved. The number of linkage groups for the mutants analyzed is still uncertain because of discrepencies in the results. More genetic analysis with other mutants might improve the data and a correlation of linkage groups and the number of chromosomes present might be possible in the future.

CHAPTER V

CONCLUSION -- AN EVOLUTIONARY VIEW

On an evolutionary scale, algae are probably the oldest eucaryotic photosynthetic organisms. Morphologically they have changed very little in over a billion years and were also probably the first organisms to reproduce sexually (Schopf, 1970). It is of interest, then, to see what controls the sexual reproduction of such a primitive organism and speculate on the advantages of developing and retaining the ability to reproduce sexually.

From the results in Chapter II one can conclude that the sexuality of Eudorina elegans is controlled by nutrients in the medium - namely a deficiency of NO_3^- and an abundance of Ca^{++}. This study, as well as an investigation by Goldstein and students (Szostak, et al., 1973), shows that conditioned medium (CM) can also induce sexuality in the male strain with the conclusion that a hormonal substance is present in CM which induces sperm production. The idea has been presented here that the nutrient requirements control the production of the hormone which in turn induces sexuality in the male. Induction of sexuality is much less understood in the female strain because gametogenesis is not observed.

Sexuality in the female E. elegans seems to be dependent on the size of the vegetative cells which is controlled to a certain extent by the nutrients in the medium.

Other factors affecting sexuality in E. elegans seem to be the necessity of light and enhancement by CO_2 of zygote production. Zygote production and germination seem to be able to take place in varying environmental conditions. Certainly being able to sexually reproduce in a variable environment has evolutionary advantages. The zygospore of E. elegans is able to withstand temperatures from below $0^{o}C$ to over $40^{o}C$ and can still germinate.

The main advantage of sexual reproduction is very often given by biologists as a mode of genetic exchange which will increase variation in an organism and possibly allow it to become more adaptable to a changing environment (Herskowitz, 1965, p. 112). However, is this the main advantage for sexual reproduction in a primitive organism like E. elegans? The immunological studies of Chapter III show that the mating strains are remarkably similar on the molecular level. Studies by Coleman (1963) and Brown and Walne (1967) show that isolates of Pandorina and Chlamydomonas only mate with those strains that are immunologically similar. Many mutants

of E. elegans not described here cannot be sexually induced to mate with the wild type strain or with other mutants derived from the same 1200/1201 stock. If genetic exchange is the main advantage for sexual reproduction in a primitive alga like Eudorina, one would expect sexual reproduction to occur more often between different isolates and between mutants from the same strain.

It is more reasonable that the main evolutionary advantage for sexual reproduction in E. elegans is not genetic exchange, but the ability to withstand a variable or hostile environment in the zygospore state. When conditions are again optimum for vegetative growth, the zygote will germinate and vegetative growth will again occur. Certainly there are limitations in applying laboratory results to the outside environment, however, environmental observations of E. elegans have been made by Iyengar (1937) which might also support this view. Every year he observed E. elegans growing in rainwater pools in Madras, India during the summer monsoon season. Towards the end of the season in September, sperm clumps and egg cells were generally formed. These gametes could then produce zygotes which remain dormant, but viable, through the winter ready to germinate during the

next summer monsoon season when conditions for vegetative growth are good and enough rain assures any inhibitors of germination would be washed away from the zygote. This type of annual life history is dependent on sexual reproduction in Eudorina. Genetic exchange seems to be of little importance to an autotrophic alga like Eudorina.

As noted previously with the exception of Mishra's work, most of the formal genetic analysis in Phycology has been with Chlamydomonas species. Goldstein first showed a 1:1 segregation of mating type alleles in Eudorina demonstrating meiosis does occur upon zygote germination. Mishra's work with a few drug-resistant mutants showed a system of uniparental inheritance exists in E. elegans as well. The present study has extended the genetics of Eudorina by using several different kinds of mutant phenotypes as markers, all of which are inherited in a Mendelian fashion. Linkage maps for these mutations have been calculated where possible.

Genetic analysis of E. elegans should be continued using other mutants that are sexually viable to see exactly how many loci regulate production of chlorophyll, morphology, or the ability to synthesize necessary substrates like acetate or nicotinamide. A more complete

genetic analysis may help to better explain genetic regulation in Eudorina, the question of redundancy of chromosomes, and a correlation of linkage groups with the number of chromosomes present. The sexuality and genetics of any of the algal systems are just beginning to be understood.

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Appendix I

Media for Eudorina elegans

Soil water extract, SE (Mishra, 1967)

To 1000 ml of glass-distilled H_2O add:

soil	62 ml
peat	31 ml
sand	31 ml
$CaCO_3$	100 mg

Steam mixture for 1 hour on 2 consecutive days.

Filter soil water using a Buchner funnel and No. 1 Whatman filter paper.

Autoclave.

Volvox medium, VM (Darden, 1966)

For each 1000 ml of medium the following amounts of stock solutions were added to 945 ml of glass-distilled H_2O:

Amount	Stock Solution	Stock Concentration
10 ml	$Ca(NO_3)_2 \cdot 4H_2O$	1.8g/100 ml
10 ml	$MgSO_4 \cdot 7H_2O$	0.40g/100 ml
10 ml	Na_2 glycerolphosphate-$5H_2O$	0.50g/100 ml
10 ml	KCl	0.50g/100 ml
10 ml	Glycylglycine	5.00g/100 ml
3 ml	P IV metal solution	see below

Adjust pH to 7.0 with 1 N NaOH

P IV metal solution

Add the following amounts of salts and chelating agent to 500 ml of glass-distilled H_2O:

$FeCl_3 \cdot 6H_2O$	0.097 g
$MnCl_2 \cdot 4H_2O$	0.041 g
$ZnCl_2$	0.005 g
$CoCl_2 \cdot 6H_2O$	0.002 g
Na_2MoO_4	0.004 g
Na_2EDTA	0.750 g

Bristol's minimal medium, BM (Cain, 1964)

For each 1000 ml of medium the following amounts of stock solutions were added to 937 ml of glass-distilled H_2O:

Amount	Stock Solution	Stock Concentration
10 ml	$NaNO_3$	0.294 M
10 ml	$CaCl_2$	0.025 M
10 ml	$MgSO_4 \cdot 7H_2O$	0.0305 M
10 ml	K_2HPO_4	0.043 M
10 ml	KH_2PO_4	0.129 M
10 ml	NaCl	0.060 M
1 ml	Gaffron's Trace Element Solution	see below
1 ml	EDTA solution	see below
1 ml	Ferrous Sulfate Solution	see below

Adjust pH to 6.8-7.0.

I. Gaffron's Trace Element Solution

To 1000 ml of glass-distilled H_2O add:

H_3BO_3	3.100 g
$MnSO_4 \cdot 4H_2O$	2.230 g
$ZnSO_4 \cdot 7H_2O$	0.287 g
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.088 g
$Co(NO_3)_2 \cdot 4H_2O$	0.146 g
$NaWO_4 \cdot 2H_2O$	0.033 g
$CuSO_4 \cdot 5H_2O$	0.125 g
KBr	0.119 g
KI	0.083 g
$Cd(NO_3)_2 \cdot 4H_2O$	0.154 g
$NiSO_4(NH_4)_2SO_4 \cdot 6H_2O$	0.198 g
$VOSO_4 \cdot 2H_2O$	0.020 g
$Al_2(SO_4)_3K_2SO_4 \cdot 24H_2O$	0.474 g
$Cr(NO_3)_3 \cdot 7H_2O$	0.037 g

Adjust pH to about 7.5 with HCl after autoclaving.

II. EDTA Solution

To 1000 ml of glass-distilled H_2O add:

EDTA	50.0 g
KOH	31.0 g

III. Ferrous Sulfate Solution

To 1000 ml of glass-distilled H_2O add:

$FeSO_4 \cdot 7H_2O$	4.98 g
H_2SO_4 (10 N)	1.0 ml

Bristols Complete Medium, BC (Kemp and Wentworth, 1971)

For each 1000 ml of medium combine:

Bristols Minimal Medium	800 ml
Modified Euglena Medium	200 ml

Modified Euglena Medium:

To 980 ml of glass-distilled H_2O add:

Difco yeast extract	2.0 g
Sodium acetate	1.0 g
Difco beef extract	1.0 g
$CaCl_2$	0.01 g
Casamino Acid Solution	20. ml

Casamino Acid Solution:

To 1000 ml of glass-distilled water add: 100.0 g Casamino acid hydrolysate (Difco vitamin free).

Add 0.1 g of charcoal to decolorize.

Filter using a millipore filter.

Autoclave.

Appendix II

Solutions Used in Immunology

1) Buffered sucrose solution

10% (w/v) sucrose

0.008 M $MgCl_2$

0.01 M Tris

Adjust pH to 7.5 with thioglycollate.

2) 0.15 M NaCl in buffered solution(.01 M PO_4 buffer)

NaCl	8.7 g
$NaH_2PO_4 \cdot H_2O$	0.2208 g
$Na_2HPO_4 \cdot 7H_2O$	2.252 g
H_2O	1000 ml

Adjust pH to 7.5.

Appendix III

Mutant-Mutant Crosses

Cross	Progeny			Chi square value	Probability of deviation from independent assortment	Linkage distance
	Phenotype	Number	Total			
1200-2 x 1201-3	wildtype light green	319 1320	1639	26.80	p<.01	39.0-32.6
1200-2 x 1201-9	wildtype light green pale	61 230 241	532	52.27	p<.01	23.0-20.6
1200-2 x 1201-11	wildtype light green	156 748	904	28.90	p<.01	34.5-29.4
1200-2 x 1201-21	wildtype light green granular light-granular	114 135 115 101	465	5.08	p>.05	NL
1200-2 x 1201-22A	wildtype light green	87 334	421	4.21	.25<p<.05	probably NL
1200-2 x 1201-32	wildtype light green pale	138 702* 565*	1405	172.62	p<.01	19.6-17.9
1200-2 x 1201-44	wildtype light green pale	73 174 192	439	17.39	p<.01	33.3-28.5
1200-2 x 1201-46	wildtype light green	145 603	748	12.5	p<.01	38.8-32.5

1200-3 x 1201-11	wildtype light green	31 130	161	2.83	p>.05	NL
1200-3 x 1201-21	wildtype light green granular light-granular	18 20 18 11	67	2.84	p>.05	NL
1200-3 x 1201-22A	wildtype light green	0 2	2	NC	-	-
1200-3 x 1201-32	wildtype light green pale	0 2 1	3	NC	-	-
1200-3 x 1201-46	wildtype light green	1 2	3	NC	-	probably NL
1200-7 x 1201-3	wildtype light green	3 6	9	NC	-	probably NL
1200-7 x 1201-9	wildtype light green pale	8 45 54	107	18.53	p<.01	15.0-13.9
1200-7 x 1201-13	wildtype small dark light green	1 5 2	8	NC	-	probably NL
1200-7 x 1201-21	wildtype light green granular light-granular	124 132 145 113	514	4.24	p>.05	NL
1200-7 x 1201-22A	wildtype light green	12 55	67	1.80	p>.05	NL

1200-7 x 1201-32	wildtype light green pale	5 8 14	27	NC	-	probably NL
1200-7 x 1201-44	wildtype light green pale	3 17 19	39	NC	-	-
1200-7 x 1201-46	wildtype light green	24 104	128	2.67	p>.05	NL
1200-9 x 1201-7	wildtype light green pale	8 40 34	82	10.75	p<.01	19.5-17.8
1200-9 x 1201-8	wildtype pale	3 4	7	NC	-	probably NL
1200-9 x 1201-11	wildtype light green pale	2 16 26	44	NC	-	-
1200-9 x 1201-22A	wildtype light green pale	4 8 6	18	NC	-	probably NL
1200-9 x 1201-32	wildtype pale	15 42	57	0.05	p>.05	NL
1200-9 x 1201-40	pale	3	3	NC	-	-
1200-9 x 1201-44	wildtype pale	4 54	58	10.14	p<.01	13.8-12.8
1200-9 x 1201-46	wildtype light green pale	32 147 176	355	51.54	p<.01	18.0-16.6

1200-11 x 1201-2	wildtype light green	262 1317	1579	59.52	p<.01	33.2-28.4
1200-11 x 1201-3	wildtype light green	71 184	255	1.10	p>.05	NL
1200-11 x 1201-7	wildtype light green	73 147	221	7.82	p<.01**	NL
1200-11 x 1201-8	wildtype light green clumpy	4 8 4	16	NC	-	probably NL
1200-11 x 1201-9	wildtype light green pale	42 166 196	404	48.92	p<.01	20.8-18.8
1200-11 x 1201-21	wildtype light green granular light-granular	26 138 167 30	361	176.50	p<.01	15.5
1200-11 x 1201-22A	wildtype light green	406 911	1317	23.85	p<. 01**	NL
1200-11 x 1201-44	wildtype light green pale	21 70 71	162	12.53	p<.01	25.9-23.0
1200-11 x 1201-46	wildtype light green	64 198	262	0.045	p>.05	NL
1200-11 x 1201-61	wildtype	1	1	NC	-	probably NL
1200-13 x 1201-9	wildtype small dark pale	138 56 185	379	67.48	p<.01**	NL

1200-13 x 1201-11	wildtype small dark light green	18 34 38	80	9.65	p<.01	45.0-36.8
1200-13 x 1201-21	small dark granular	251 271	522	175.0	p<.01	0
1200-13 x 1201-22A	?? ***	380	380	NC	-	-
1200-13 x 1201-44	wildtype pale small dark	76 97 29	202	22.19	p<.01**	NL
1200-13 x 1201-46	wildtype light green small dark	48 69 39	155	3.37	p>.05	NL
1200-21 x 1201-2	wildtype light green granular light-granular	2 3 5 1	11	NC	-	probably NL
1200-21 x 1201-3	light green	1	1	NC	-	-
1200-21 x 1201-9	wildtype pale granular pale-granular	11 17 13 11	52	1.85	p>.05	NL
1200-21 x 1201-13	wildtype small dark granular	54 135 118	307	10.25	p<.01	35.2-30.0
1200-21 x 1201-22A	wildtype light green granular light-granular	45 49 61 50	205	2.74	p>.05	NL

1200-21 x 1201-44	wildtype pale granular pale-granular	1 1 1 3	6	NC	-	probably NL
1200-21 x 1201-46	wildtype light green granular light-granular	49 92 49 51	241	22.35	p<.01**	NL
1200-22A x 1201-2	wildtype light green	181 557	738	0.088	p>.05	NL
1200-22A x 1201-3	wildtype light green	77 213	290	0.37	p>.05	NL
1200-22A x 1201-9	wildtype light green pale	1 5 5	11	NC	-	probably NL
1200-22A x 1201-11	wildtype light green	240 412	652	3.56	p>.05	NL
1200-22A x 1201-13	wildtype light green small dark light-grainy	10 49 40 10	109	45.17	p<.01	18.4
1200-22A x 1201-21	wildtype light green granular light granular	195 204 211 153	763	15.12	p<.01	45.6-51.1
1200-22A x 1201-32	wildtype light green pale	29 75 81	185	8.84	.01<p<.05	31.3-24.8

1200-22A x 1201-44	wildtype light green pale	10 26 17	53	3.10	p>.05	NL
1200-22A x 1201-46	wildtype light green	32 183	215	11.73	p<.01	29.8-25.9
1200-32 x 1201-22A	wildtype light green pale	0 5 6	11	NC	-	-
1200-44 x 1201-7	wildtype light green pale	10 92 86	188	39.09	p<.01	10.6-10.1
1200-44 x 1201-9	wildtype pale	35 1195	1230	321.98	p<.01	5.7-5.5
1200-44 x 1201-11	wildtype light green pale	34 50 70	154	4.16	p>.05	NL
1200-44 x 1201-21	wildtype pale granular pale-granular	91 122 100 47	360	33.04	p<.01**	NL
1200-44 x 1201-22A	wildtype light green pale	2 1 2	2	NC	-	probably NL
1200-44 x 1201-32	wildtype pale	31 87	118	0.10	p>.05	NL

1200-44 x 1201-46	wildtype light green pale	51 200 225	476	53.56	p<.01	21.4-19.4
1200-44 x 1201-61	wildtype pale nic⁻(dark)	6 6 1	13	NC	-	probably NL
1200-46 x 1201-9	wildtype light green pale	10 41 57	108	17.43	p<.01	18.5-17.0
1200-46 x 1201-11	wildtype light green	91 338	429	3.28	p>.05	NL
1200-46 x 1201-13	wildtype light green small dark	57 72 57	186	2.41	p>.05	NL
1200-46 x 1201-21	wildtype light green granular light-granular	44 93 110 58	305	36.63	p<.01	33.4
1200-46 x 1201-22A	wildtype light green	122 384	504	0.17	p>.05	NL
1200-46 x 1201-44	wildtype light green pale	9 51 55	115	18.28	p<.01	15.6-14.5

NC -- chi square not calculated, insufficient data

NL -- not linked

* -- difference in phenotype not distinct

** -- significant deviation not due to linkage

*** -- phenotypes not differentiated due to contamination

Curriculum Vitae

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