*CHEMICAL ALTERATION OF CROSSING-OVER FREQUENCY IN CHLAMYDOMONAS**

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Mazia and Ambrose and Gopal-Ayengar have recently suggested that chromosomes are composed of particulate "macromolecular complexes of nucleic acid and proteins. . .linked together by bridges of divalent ions (Ca, Mg, or both)"[1] or by hydrogen bonds.[2] This hypothesis was based on various lines of evidence: that chromosomes disperse when treated with chelating agents;[1] the observations of Steffensen[3] that deficiencies in either calcium or magnesium increase the frequency of chromosome breaks in *Tradescantia;* and Williamson and Gulick's[4] finding that mammalian cell nuclei contain unusually large amounts of calcium and magnesium. In this connection it is of interest that Jungner[5] has found relatively high concentrations of magnesium in purified nucleic acids from several sources and has observed that the magnesium content varied within fairly narrow limits, apparently characteristic of the various nucleic acids examined.

Genetic crossing over[6] would be expected to be intimately related to chromosome structure. Accordingly, an investigation was undertaken of the effects of calcium and magnesium and of treatment with a chelating agent on crossing over in *Chlamydomonas.* This haploid uninucleate green alga is in some ways perhaps better suited experimentally[7, 8] for such studies than is *Drosophila,* in which Levine[9] has recently demonstrated modification of crossing-over frequency by changes in calcium level.

Methods.—Six growth-factor-requiring strains of *Chlamydomonas reinhardi* Stock 137C, representing three pairs of linked genes, were used for the cross-over frequency studies to be reported. The *arg*-1 strain requires arginine, citrulline, or ornithine,[7] and the *arg*-2 strain utilizes arginine only; the two genes responsible for the deficiencies are about 6 map units apart. The *pab*-1 strain will grow only if supplied *p*-aminobenzoic acid, and its requirement is controlled by a gene which is roughly 23 map units from the gene responsible for the nicotinamide requirement of the *nic*-5 strain. The intact thiamine molecule is necessary for growth of the *thi*-1 mutant, and acetate is required for growth of mutant strain *ac*-157b. The *thi*-1 locus is 14 map units from the *ac*-157b locus. For further details on these strains and on culture methods and media see Eversole.[8]

As a preliminary screening technique for exploring conditions and chemical treatments which might influence crossing-over frequency, *in situ* germination of zygotes as described by Eversole[8] was employed, using crosses of mutant strains *arg*-1 by *arg*-2. When treatment altering crossing-over frequency were found, more precise genetic analyses were performed, using the manual manipulation procedure of Ebersold[7] for the isolation of zygotes and in some cases isolation of their four meiotic products.

Three different chemical treatments of cells for one or more hours just prior to mating were investigated for their effects on crossing over. These were (1) ethylenediamine tetraacetic acid (EDTA) extractions, (2) $MnCl_2$ treatment,[10] and (3)

an incubation in high concentrations of calcium and magnesium subsequent to either EDTA or $MnCl_2$ treatment. Yeast extract–acetate agar grown cells were extracted with 10^{-4} *M* EDTA in Petri plates at 20° C. with a 100-watt light bulb at one side. They were permitted to swim under these phototactic conditions for 24–48 hours, except that in certain experiments with *arg*-1 and *arg*-2 the extraction was terminated after 6 hours. At the end of the extraction period the cells were immediately mated on the surface of minimal agar plates. The procedure of Demerec and Hanson[10] for $MnCl_2$ treatment of *Escherichia coli* was followed for cross-over experiments with *Chlamydomonas*. Cells grown on yeast extract–acetate agar were suspended in water for 1–2 hours, in which time they formed flagella and accumulated phototactically. They were centrifuged and washed with 0.019 *M* sodium chloride, the highest concentration which *Chlamydomonas* will tolerate, and incubated for 1 hour in 0.04 per cent $MnCl_2$. Then the cells were mated immediately in the usual manner on minimal agar. The number of reverse mutations produced by this treatment and viability were determined by plate counts on minimal and on supplemented agar media. Treatment was varied in certain experiments by omitting the sodium chloride wash and suspending the cells directly in $MnCl_2$. Under these conditions the cells did not become motile or accumulate phototactically within 1 hour; they were therefore left in $MnCl_2$ for $2^1/_2$–4 hours.

Certain treatments were followed by incubation of the cells in high concentrations of calcium and magnesium. Following the EDTA extractions, 5 ml. of a solution containing 200 p.p.m. Ca ($CaCl_2$) and 200 p.p.m. Mg ($MgCl_2$) were added to 5 ml. of EDTA containing the extracted cells, and the mixture was allowed to stand for several hours before mating. Following the $MnCl_2$ treatment, the cells were centrifuged and resuspended in the same Ca-Mg solution for several hours and then were mated.

Intracellular concentrations of calcium and magnesium were determined on cells with and without extraction with EDTA. The cells were grown for 7–9 days in the light at 25° C. on the surface of yeast extract–acetate agar medium in 500-ml. Erlenmeyer flasks. The cells were scraped off the surface and placed either in distilled water or in 10^{-4} *M* EDTA in Petri plates and allowed to stand at 20° C., with light coming from one side. At the appropriate time a small sample of cells was taken for mating to determine cross-over frequency. The remaining cells were washed twice and dried overnight at 80° C., and the dry weight was determined. Sufficient cells were used to give about 200 mg. of dry cells per crucible. The dry cells were ashed in an oven at 1500°–1550° F. for 6–7 hours. The prolonged ashing time was used as a means of eliminating amounts of sodium, potassium, and phosphate which would interfere with subsequent calcium analyses. After the crucible had cooled in a desiccator and the weight had been determined, the ash was dissolved in concentrated nitric acid and the solution evaporated to dryness. The residue was dissolved in distilled water and diluted to 10 ml., and aliquots were analyzed for calcium and magnesium. The direct EDTA titration method of Gehrke, Affsprung, and Lee[11] for magnesium determination was adapted for use on ashed cells. Oxalate precipitation of calcium and zinc was used to eliminate the small amounts of these interfering substances, and the concentrations of EDTA used for titrations were more dilute (*ca.* $10^{-5}M$). Calcium was determined by direct-flame photometry, according to Kingsley and Schaffert.[12]

Results.—Suitable conditions for EDTA extraction and $MnCl_2$ treatment were established by following recombination by *in situ* germination. Zygotes produced at the same time, but kept in the dark, were then manipulated to fresh minimal agar plates, and in some cases the four meiotic products were separated after zygote germination. Results of experiments with whole isolated zygotes from crosses of strains *arg*-1 by *arg*-2 are shown in Table 1. The *F*-value is a convenient approximate measure of recombination:

$$F = \frac{\text{No. recombination zygotes} \times 100}{\text{Total no. zygotes}}$$

Recombination zygotes (tetratype and nonparental ditype)[13] contain wild-type recombinants and hence grow as wild-type colonies on minimal medium. The values of *F* for *arg*-1 by *arg*-2 zygotes under normal conditions have varied between 6 and 13 in seven experiments involving over one thousand zygotes. When extractions with EDTA were of 24 or more hours' duration, increased *F*-values be-

TABLE 1

RECOMBINATION IN WHOLE ZYGOTES* FROM CROSSES OF *arg*-1 BY *arg*-2

TREATMENT	WILD TYPES	TOTAL	*F*	Mg†		Ca†	
				arg-1	*arg*-2	*arg*-1	*arg*-2
None	...	...	6–13	402	498	645	602
				386	398	505	629
EDTA (39 hr.)	110	145	76.0	164	153	404	523
EDTA (47 hr.)	69	109	63.3	102	139	502	486
EDTA (25 hr.)	49	83	59.0	224	185	490	523
EDTA (6 hr.)	27	90	30.0	...	...	...	...
Then Ca-Mg	4	62	6.4	...	...	...	...
EDTA (6 hr.)	27	88	30.7	...	...	...	...
Then Ca-Mg	5	69	7.3	...	...	...	...
(NaCl) $MnCl_2$	139	179	77.7	...	...	...	...
(NaCl) $MnCl_2$	55	91	60.5	...	...	...	...
(NaCl) $MnCl_2$	48	64	75.0	...	...	...	...
$MnCl_2$	32	121	26.4	...	...	...	...
Then Ca-Mg	9	71	12.6	...	...	...	...
$MnCl_2$	36	99	36.4	...	...	...	..
Then Ca-Mg	8	76	10.5	...	...	...	...

* Zygotes manipulated to fresh agar plates.

† Results expressed in micrograms of Ca or Mg per gram dry weight of cells.

tween 59 and 76 were obtained in three experiments involving 337 zygotes. Following prolonged EDTA extractions and treatment with Ca and Mg, the cells were no longer sexually functional. Zygotes could be produced, however, with EDTA extractions of only 6 hours, followed by Ca-Mg treatment. In these cases *F*-values for the EDTA extraction alone (30.0 and 30.7) were higher than in controls but lower than in experiments involving longer extraction times. Following the Ca-Mg treatment, the *F*-values were 6.4 and 7.3. Since the crossing-over frequency could be restored to the control level by treatment with calcium and magnesium, the EDTA effect appears to be correlated with deficiencies of these metallic ions.

$MnCl_2$ treatment is similar in its effect to that of EDTA. The values of *F* varied from 60.5 to 77.7 in three experiments in which more than 95 per cent of the cells survived the treatment and no reverse mutations were recovered in 10^5 cells. Therefore, all the wild types recovered from crosses are considered to be genetic recombinants and not reverse mutations. After NaCl-washed, $MnCl_2$-treated

cells were incubated in a calcium and magnesium solution, zygote formation failed in the case of *arg*-1 by *arg*-2; however, a reversal of the $MnCl_2$ effect was possible when the NaCl wash was omitted. In this case F was also lower (26.4 and 36.4), but Ca-Mg treatment returned the values essentially to the control range (12.6 and 10.5).

Table 2 shows the results of tetrad analysis of zygotes from some of the *arg*-1 by *arg-2* experiments. These findings confirm the results given in Table 1.

TABLE 2

TETRAD ANALYSIS* OF ZYGOTES FROM CROSSES OF *arg*-1 BY *arg*-2

Treatment	PD	T	NPD	F	Map Distance	Expected NPD†
None	198	23	3	11.6	6.5	0.31
EDTA (39 hr.)	21	18	8	55.2	36.2	1.08
EDTA (25 hr.)	14	23	9	69.5	44.6	1.91
EDTA (6 hr.)	30	15	2	36.2	20.2	0.67
Then Ca-Mg	41	4	1	10.9	6.5	0.05
(NaCl) $MnCl_2$	15	23	10	68.8	44.8	1.82
(NaCl) $MnCl_2$	34	38	21	63.5	43.0	2.42
(NaCl) $MnCl_2$	10	8	3	58.0	33.4	0.48
$MnCl_2$	29	11	2	30.9	17.9	0.42
Then Ca-Mg	47	4	0	7.9	3.9	0.04

* PD, parental ditype; T, tetratype; NPD, nonparental ditype.

† Calculated as NPD = $T^2/8\ (1 + 2T/3)$; see H. P. Papazian, *Genetics*, **37**, 175, 1952.

Intracellular concentrations of calcium and magnesium were determined in the three EDTA experiments in which the extractions were longer than 24 hours and, for comparison, in two experiments with untreated controls. These results are included in Table 1 and show that magnesium, but not calcium, was significantly lower. On this basis the EDTA effect is associated with a magnesium deficiency. Calcium and magnesium were both significantly lower following prolonged EDTA extraction in cases in which zygote formation failed. Lewin[14] has reported that calcium is required for zygote formation in *C. moewusii*.

Experiments with two additional pairs of gene loci, apparently in different linkage groups, are summarized in Table 3. The cross-over frequency between *pab*-1

TABLE 3

RECOMBINATION IN WHOLE ZYGOTES* FROM CROSSES OF *pab*-1 BY *nic*-5 AND *thi*-1 BY *ac*-157b

TREATMENT	*pab*-1 BY *nic*-5			*thi*-1 BY *ac*-157b		
	Wild Types	Total	F	Wild Types	Total	F
None			38–44			25–34
EDTA (24 hr.)	54	88	61.2	29	85	34.1
Then Ca-Mg	38	89	42.7	28	90	31.1
EDTA (26 hr.)	56	88	63.6	26	93	27.9
Then Ca-Mg	42	100	42.0	30	95	31.6
(NaCl) $MnCl_2$	62	92	67.4	23	82	28.0
Then Ca-Mg	33	90	36.7	27	90	30.0
(NaCl) $MnCl_2$	53	82	64.8	26	92	28.3
Then Ca-Mg	28	92	30.4	29	97	29.9

* Zygotes manipulated to fresh agar plates.

and *nic*-5 (F = 38–44) is almost four times greater than that between *arg*-1 and *arg*-2; the effect of either EDTA or $MnCl_2$ treatment would therefore be expected to be less. However, both chemical treatments significantly increased the cross-over frequency. The frequency of recombination between *thi*-1 and *ac*-157b (F =

25–34), although initially less than between *pab*-1 and *nic*-5, was not significantly altered by either EDTA or $MnCl_2$.

The data in Table 2 on crosses between *arg*-1 and *arg*-2 suggest an excess of four-strand double crossovers, as calculated from the observed frequency of tetratypes (T) by equation (5) of Papazian.[15] The appearance of nonparental ditypes appears to be independent of EDTA or $MnCl_2$ treatments. Similar results have been reported for other crosses of *C. reinhardi* involving the *arg*-1 and *arg*-2 linkage group (Ebersold[7]) and for crosses in which the sex locus of *Neurospora crassa* was used as a marker (Barratt *et al.*[13]). Such excesses above the expected ratio of NPD:T could arise from negative chiasma interference, from positive chromatid interference, or from single exchanges at the two-strand stage. Moewus[16] has reported crossing over at the two-strand stage in 100 per cent of the cases when *C. eugametos* zygotes are germinated at low temperatures. However, in the experiments reported here, two-strand-stage crossing over in less than 1 per cent of the cases could account for the observed excesses of nonparental ditypes.

The results reported here show clearly that the ionic environment significantly affects crossing-over frequency in *Chlamydomonas*. The effect of EDTA extraction, shown to result in decreased levels of Ca and Mg, increases crossing-over frequency, in a manner qualitatively similar to that reported by Levine[9] for *Drosophila*, although the quantitative effect in *Chlamydominas* is considerably greater. There would appear to be an additional difference in regard to crossing over, in that the effects of variations of Ca level seem greater in *Drosophila*[9] and of Mg level in *Chlamydomonas*. The difference in behavior of the loci examined in *Chlamydomonas* suggests that different regions of chromosomes of this microorganism may differ in susceptibility to crossing over in relation to the cationic environment. Further work will be required to determine the extent and significance of this variability.

The ionic environment could affect crossing over indirectly, via physiological routes. However, the results reported here and those reported by Levine[9] are consistent with a role of bivalent cations in chromosome structure as proposed by Mazia.[1]

A satisfactory explanation of the $MnCl_2$ effect is more difficult to formulate than that of EDTA. However, a mechanism of action related to that of EDTA is suggested by the reversal of the effects of both chemicals by calcium and magnesium and by the similar responses of different chromosome regions to both chemicals. It is possible that the $MnCl_2$ effect is due to the replacement of some of the Mg in the chromosome by Mn. The chelates of magnesium and manganese are quite similar chemically. The stability constants of the chelates of the two metals are roughly equivalent, but manganese has a slightly greater affinity than magnesium for most organic chelating agents[17] and perhaps for chromosomal constituents.[18]

Summary.—Variations in crossing-over frequency have been shown to follow treatment of cells of biochemical mutant strains of *C. reinhardi* with a chelating agent or with $MnCl_2$. The frequency of crossing over was increased by these treatments in two of three chromosome intervals tested. The effects of both agents were reversed by subsequent incubation of treated cells in high concentrations of calcium and magnesium ions. It is suggested that the action of $MnCl_2$ may involve the replacement of Mg by Mn.

The sensitivity of crossing over in *C. reinhardi* to ionic environment is consistent with the theory that chromosomes are composed of macromolecular unit particles linked together by divalent ions.

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A FURTHER ANALYSIS OF THE FORKED LOCUS IN DROSOPHILA MELANOGASTER

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One question posed by the discovery of pseudoallelism is whether crossing over may be utilized to define the limits of the gene. Alternatively, this question may be stated as follows: Are pseudoallelic mutants divisible by crossing over into a discontinuous array just as are linked, nonallelic mutants, or do pseudoalleles represent a continuous array of chromosomal sites within which crossing over occurs? The data from *Drosophila* are consistent with the first-stated view. However, the putative cases of pseudoallelism recently described in bacteriophage by Benzer[1] and in *Salmonella typhimurium* by Demerec *et al.*[2] suggest that pseudo-