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Effect of Zn^{2+} on Bacterial Conjugation: Inhibition of Mating Pair Formation

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Zn^{2+} at 10^{-3} M has been found to block the formation of mating pairs between Hfr and F^- strains of *Escherichia coli* as observed both by light microscopy and by Coulter counter measurements. Kinetic studies show that Zn^{2+} reduces the fertility of the male and that its effect disappears within 2 min after Zn^{2+} has been removed from the medium. Short treatments of female cells with Zn^{2+} have no detectable effect on their ability to form mating pairs. Later steps in the mating process such as mobilization of the male chromosome, transfer of the chromosome to the female, or its integration into the female chromosome seem not to be affected by 10^{-3} M Zn^{2+}.

Many investigators have shown that the organelles known as F pili are necessary for the fertility of male bacteria (4-10, 14, 16, 18, 21), and, by observing individual mating pairs, we ourselves have shown that F pili may indeed serve as conveyers for deoxyribonucleic acid (DNA) transfer between pairs that are separated by a number of micrometers (22). It is known that both male-specific spherical ribonucleic acid (RNA) phages, that attach to the sides of F pili, and filamentous male-specific DNA phages, that attach to the tips of F pili (6), reduce the numbers of recombinants formed by mixtures of male and female bacteria (16, 18, 21). The simplest explanation of these effects would be that these phages inhibit the formation of mating pairs by blocking the attachment of F pili to receptor sites on the surfaces of female cells. However, other explanations are possible, for example, infection by these phages might render males or females infertile by blocking other later steps necessary for the appearance of recombinants.

The observation of Tzagoloff and Pratt (27) that 10^{-3} M Zn^{2+} prevents the adsorption of the filamentous phage M13 together with the association of Zn with DNA polymerase activity (24) led us to test the effect of Zn^{2+} on bacterial mating. We have found that nonlethal concentrations of Zn^{2+} indeed do inhibit the formation of mating pairs but they have no detectable effect on the later stages in the mating process such as chromosome mobilization in the male, the transfer of the chromosome to the female, or its integration into the female chromosome to form recombinants.

MATERIALS AND METHODS

Bacterial strains. HfrW3011, F^-W1-3, and F^- W1-4, all derivatives of *Escherichia coli* K-12 strains (22), were used in these investigations as donor and recipients. HfrW3011 is an Hfr of the Cavalli type [entry times of *lac*, *leu*, and *str*: 10, 19, and 46 min, respectively; see Taylor (26) for definition of gene symbols and the standard map of *E. coli*] and is lactose-utilizing (Lac^+), leucine-independent (Leu^+), proline-independent (Pro^+), and streptomycin-sensitive (Str^s). Strain W1-3 and its derivative W1-4 are F^-, lactose nonutilizing (Lac^-), and streptomycin-resistant (Str^R). Strain W1-3 is Leu^- Pro^+, whereas strain W1-4 is Leu^+ Pro^-. All strains were lyophilized in ampoules for storage. Their genetic characters were periodically confirmed.

Media. Bacterial strains were routinely grown and mated in nutrient broth (1% tryptone, 0.5% yeast extract, 1.0% NaCl, and 0.1% glucose, adjusted to *p*H 7 with 1 N NaOH). An indicator medium, MacConkey agar containing 100 μg of streptomycin-sulfate per ml, was used for measuring Lac^+ Str^R recombinants. For detection of Leu^+ Str^R recombinants, the M9 synthetic medium prepared by the method of Adams (1), containing appropriate nutrients except leucine, was used. Saline (0.9%), filtered through a 0.45-μm pore size membrane filter (Millipore Corp.) was used as the electrolyte for Coulter counter measurements. A stock solution of 0.1 M $ZnSO_4$ was used as the source of Zn^{2+}.

Mating methods. Unless otherwise mentioned, the following manner of mating was employed throughout the present investigation. Overnight broth cultures were diluted 100-fold into fresh broth and incubated with aeration until there were 5×10^8 to 10^9 cells/ml. To allow mating pairs to form, 2 ml each of Hfr and F^- cells were mixed in a prewarmed 25-ml Ehrlenmeyer flask. After 30 min of gentle shaking, mating pairs were separated by mechani-

cally blending the mixture for 6 sec as described by Low and Wood (20). The mixture was then diluted and plated onto appropriate solid media. Recombinant colonies were counted after 18 to 20 hr of incubation for Lac^+ Str^R and after 48 hr for Leu^+ Str^R.

The extent of mating pair formation was also measured with a Coulter counter (model B, Coulter Electronics, Sheffield, Chicago) equipped with a 30-μm aperture and a 50-μliter manometer. Hfr and F^- cultures ($\sim 4 \times 10^8$ cells/ml) were diluted 10^{-2}-fold into 10 ml of filtered saline and counted to serve for the measurement of input parental cells. Then equal volumes of Hfr and F^- cultures were mixed and samples were withdrawn gently at intervals and diluted 10^{-2}-fold into saline for measurement.

To study the kinetics of the recovery of Hfr cells from exposure to Zn^{2+}, so-called dump experiments were carried out (2). An Hfr culture, in late exponential phase, was divided into two 3-ml portions. Zn^{2+} was added to one of these to a final concentration of 10^{-3} M. The two portions were then centrifuged at 7,000 rev/min for 10 min, the supernatant fluids were discarded, and the bacterial pellets were resuspended in the remaining 0.1-ml droplet of supernatant fluid. At time (t) = 0, 3 ml of an F^- culture was then dumped onto each of the concentrated Hfr cultures to allow males to conjugate in the control culture, to dilute out the Zn^{2+} from the males in the experimental culture, and to allow them to conjugate as they recovered from their exposure to Zn^{2+}. At various short times thereafter, samples of the mixtures were gently diluted 2,000-fold into broth at 37 C to prevent further pair formation and to allow conjugating males to transfer genetic material to their mates. At t = 30 min, the diluted mixtures were blended to break mates apart and plated on MacConkey agar for detection of Lac^+ recombinants.

To see if Zn^{2+} might affect the fertility of females, analogous dump experiments were performed in which males were added to females that had been pelleted in 10^{-3} M Zn^{2+}.

To determine the effects of Zn^{2+} on later steps in conjugation, interrupted matings were performed (11): equal amounts (2 ml each) of exponentially growing male and female cells were mixed, and after a few minutes the mixture was gently diluted 1,000-fold into prewarmed fresh broth to prevent further formation of mating pairs. One sample of the diluted suspension was then made 10^{-3} M in Zn^{2+}, whereas the other served as a control. Periodically thereafter, 0.1-ml samples were diluted, blended to interrupt mating, and plated onto appropriate plates for detection of recombinants.

Experiments designed to determine the effect of Zn^{2+} on the integration of transferred genes were carried out as follows: a standard mating without Zn^{2+} as described above was performed. After 30 min the mating was interrupted by blending as usual and a sample was diluted 10^5-fold into fresh broth. This sample was then divided in two and Zn^{2+} was added to one of the resulting samples. At regular periods during incubation thereafter, samples were plated onto MacConkey agar for detection of Lac^+ Str^R recombinants. Streptomycin (100 μg/ml) was included to suppress growth of the male. Incidentally, the growth of F^- cells to which 10^{-3} M Zn^{2+} was added was found to be arrested for 60 min before growth resumed (*unpublished data*). To condition them to Zn^{2+}, F^- cells were grown overnight in 10^{-3} M Zn^{2+}, a sample was diluted 100-fold in fresh broth containing Zn^{2+}, and the cells were washed to eliminate excess Zn^{2+} and were used in the integration experiments.

RESULTS

Effect of Zn^{2+} on recombinant formation. The results of tests of various Zn^{2+} concentrations on recombinant formation are presented in Table 1. In general, the higher the concentration of Zn^{2+}, the lower the recombinant frequency until at concentrations higher than 4×10^{-3} M the cells were no longer able to form colonies. Zn^{2+} at 10^{-3} M gave a high inhibitory effect without significantly affecting the viability of cells and reduced the frequency of recombinant formation to only 1/10 to 1/500 of the frequencies of control mating mixtures to which no Zn^{2+} had been added (Table 2). Therefore, this concentration of Zn^{2+} was used throughout the rest of the experiments. Incidentally, our nutrient broth contained about 10^{-5} M Zn^{2+} as determined by atomic absorption. The effects of comparable added concentrations (10^{-3} M) of other divalent ions such as Mg^{2+} and Ca^{2+} were negligible.

Now we would like to know which steps in mating are affected by Zn^{2+}. Five steps in mating are easily recognized: (i) mating pair formation, (ii) chromosome mobilization within the male, (iii) transfer of genetic material from male to female, (iv) integration of the transferred material into the female chromosome to form one or more recombinant chromosomes, and (v) segregation of the chromosomes in daughter cells. We carried out the following experiments to see which steps are affected by Zn^{2+}.

Inhibition of mating pair formation by Zn^{2+}. When mating mixtures in broth were observed in a light microscope, many conjugating cells could be seen. If motile males were used, males towing females as much as 5 μm behind them could be observed (3, 22). When Hfr and F^- cells were mixed in the presence of Zn^{2+}, however, very few aggregates were observed. When male cell cultures alone were observed, one again had the impression that the cells without Zn^{2+} were very sticky and many cell clumps could be observed, whereas parallel cultures with Zn^{2+} contained very few cell clumps and the cells were, in general, quite indifferent to one another.

TABLE 1. *Effect of Zn^{2+} concentration on frequency of recombinant formation in 30-min mating between HfrW3011 and F⁻W1-3*

Expt no.	Recombinant	No. of recombinants per 100 males			
		No added Zn^{2+}[a]	$+10^{-4}$ M Zn^{2+}	$+5 \times 10^{-4}$ M Zn^{2+}	$+10^{-3}$ M Zn^{2+}
1	Lac⁺ Str^R	17.0	11.5		0.57
2	Lac⁺ Str^R	30.0	39.0		
	Leu⁺ Str^R	0.75	1.4		
3	Lac⁺ Str^R	39.0		22.0	
	Leu⁺ Str^R	0.79		0.39	
4	Lac⁺ Str^R	67.2			0.31
	Leu⁺ Str^R	2.0			0.0077

[a] Broth contains approximately 10^{-5} M Zn^{2+} as determined by atomic absorption methods.

TABLE 2. *Effect of Zn^{2+} on frequency of recombinant formation in 30-min mating between HfrW3011 and F⁻W1-3*

Expt. no.	Recombinant	No. of recombinants per 100 males		
		(A) No Zn^{2+}	(B) + 10^{-3} M Zn^{2+}	Ratio B/A
1	Lac⁺ Str^R	2.3[a]	0.28	0.12
	Leu⁺ Str^R	0.06	0.0054	0.15
2	Lac⁺ Str^R	40.3[a]	0.65	0.016
	Leu⁺ Str^R	0.87	0.025	0.029
3	Lac⁺ Str^R	67.2	0.31	0.0022
	Leu⁺ Str^R	2.3	0.0077	0.0033
4	Lac⁺ Str^R	17.0	0.57	0.033
5	Lac⁺ Str^R	17.0	0.16	0.0094
6	Lac⁺ Str^R	3.26	0.256	0.079
7	Lac⁺ Str^R	0.6	0.006	0.01

[a] Large spontaneous variations in recombinant frequencies obtained from day to day are common and their causes are unknown. In both "good" and "bad" experiments, 10^{-3} M Zn^{2+} drastically reduced the number of recombinants.

We have also used a Coulter counter to monitor the formation of mating pairs. The Coulter counter registers the impulses which are produced when particles suspended in an electrolyte pass through a constricted aperture where an electric current is flowing and thus change the resistance of the electrolyte in the aperture. The number of impulses observed after a known volume has passed through the aperture corresponds to the number of cells or clumps of cells in that volume, for when cells are extremely close together, as they are in a mating pair or in a clump, they register only one impulse. Therefore, as more and more cells form mating pairs, the total number of impulses decreases as a function of time. To minimize variance due to cell growth, we used cultures in late exponential phase where the number of cells per unit volume increases only slowly.

The following formulae, devised by R. Walmsley (*personal communication*), were employed in computing the concentration of mating pairs formed and the kinetics of mating pair formation. At the time of mixing (t = 0), the total number of impulses from a unit volume is

$$I_0 = F_0 + M_0 \quad (1)$$

where F_0 and M_0 represent the concentrations of input unpaired female and male cells, respectively. Then at later times, t, when mating pairs have formed, the number of impulses per unit volume will be

$$I_t = F_t + M_t + P_t + T_t + Q_t + \cdots \quad (2)$$

where F_t and M_t are the concentrations of unpaired female and male cells, respectively, and P_t, T_t, Q_t, etc. are the concentrations of mating pairs, triplets, quadruplets, etc., each of which registers a single impulse. Ignoring all but mating *pairs*, we note that

$$P_t = F_0 - F_t, \quad \text{and} \quad (3)$$

$$P_t = M_0 - M_t \quad (4)$$

When equations (1), (3) and (4) are combined,

$$I_0 = (F_t + P_t) + (M_t + P_t) = F_t + M_t + 2P_t \quad (5)$$

Then, by subtracting equation 2 from equation 5, the number of mating pairs is

$$P_t = I_0 - I_t \quad (6)$$

A typical result is presented in Fig. 1. Figure 1A is obtained by plotting I_t as a function of time, t, and Fig. 1B is obtained by plotting the percentage of males forming mating pairs ($P_t/M_0 \times 100$) against the time, t. When male and female cells were mixed without added Zn^{2+}, the counts decreased with time, indicat-

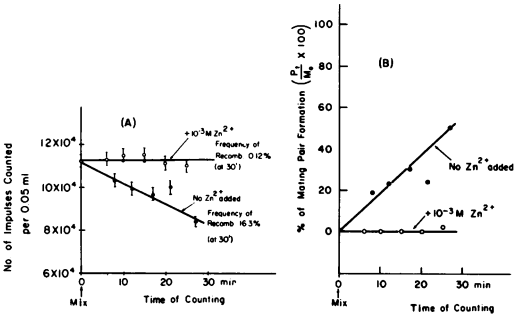

FIG. 1. *(A) Kinetics of mating pair formation with and without 10^{-3} M Zn^{2+} as measured with a Coulter counter. At time t = 0, HfrW3011 and F⁻W1-3 cultures were mixed, and, at various times thereafter, samples were diluted 1:100 and impulses were counted in the counter. They were mixed without added Zn^{2+} (●) and in the presence of 10^{-3} M Zn^{2+} (○). (B) Percentage of males forming mating pairs (see text) (ordinate) as a function of time (abscissa) without added Zn^{2+} (●) and with 10^{-3} M Zn^{2+} (○). At t = 30 min, the mixtures were diluted and blended to separate mating pairs. The samples then were plated to measure the frequency of Lac⁺ recombinant formation.*

ing the mating pairs (each giving one impulse) had formed. Whereas when they were mixed in the presence of 10^{-3} M Zn^{2+}, the number of counts remained constant, indicating that Zn^{2+} prevented the formation of mating pairs. The results confirm the observations made with the light microscope: 10^{-3} M Zn^{2+} prevents the formation of mating pairs.

We have already seen that female cells do not clump either in the presence or absence of Zn^{2+}. This result was confirmed using the Coulter counter. Clumping of male cells was also confirmed quantitatively by Coulter counter measurements as shown in Fig. 2. The number of impulses produced by the culture without added Zn^{2+} decreased steadily with time, whereas the number of impulses produced by the Zn^{2+}-enriched culture stayed constant with time. At 60 min, when the control sample (no added Zn^{2+}) was vigorously bubbled to break up the clumps before measurement, the number of impulses increased to the level of the culture containing added Zn^{2+} (the point above dotted line), strongly indicating that the decrease in the number of impulses was due to clumping. It is evident therefore that 10^{-3} M Zn^{2+} effectively eliminates the stickiness of male cells. Presumably Zn^{2+} prevents the adsorption of F pili to their adsorption sites, but the experiments reported so far do not show which elements Zn^{2+} acts on.

Does Zn^{2+} act on F pili or on receptor sites on F⁻ bacteria? To answer this question, "dump experiments" were adapted as described above. The males were treated with Zn^{2+} until mating, when the Zn^{2+} was brought. to a noneffective concentration by the addition of a large volume of female bacteria that had never been exposed to an effective concentration of Zn^{2+}. After various times, t, had been allowed for pair formation, the mating mixtures were gently diluted to avoid separating mating pairs from each other and to prevent further pair formation. Genetic transfers between pairs that had already formed were allowed to continue for 30 min. The diluted mixtures were then plated to determine the numbers of recombinants formed, N_t. Control dilutions were also made of males that had not been exposed to Zn^{2+} and plated to count the numbers of recombinants, N'_t. The ratio N_t/N'_t thus served as a measure of the persistence of the effect of Zn^{2+} on the males. As shown in Fig. 3 where N_t/N'_t is plotted against the time of dilution, t, the recovery is fairly rapid: when mating pairs were allowed to form for more than 2 min after mixing, the differences between Zn^{2+}-treated males and controls became insignificant. To estimate the rate of recovery, we assume that Zn^{2+} dissociates from the treated males, $M:Zn^{2+}$ accordingly to a monomolecular reaction with a rate constant k_1:

$$M:Zn^{2+} \xrightarrow{k_1} M + Zn^{2+}$$

The now potent males, M, then encounter females, F, in a second reaction to form mating pairs, P, with a rate constant, k_2:

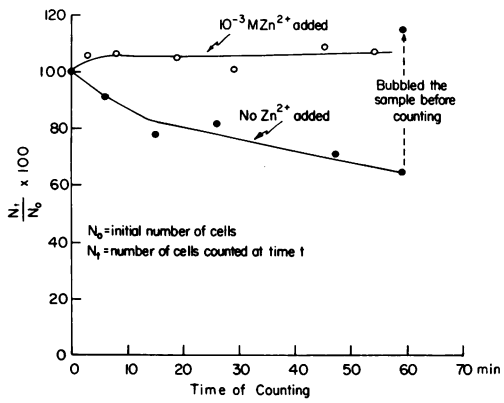

FIG. 2. *Coulter counter measurements of the clumping of male bacteria (HfrW3011) with (○) and without (●) 10^{-3} M Zn^{2+}. At t = 0, male cultures were shaken to break up clumps that might have formed. At t = 60 min, the sample without Zn^{2+} was agitated again by bubbling.*

$$M + F \xrightarrow{k_2} P$$

Since only a few per cent of the females become fertilized, we can assume that their concentration remains constant and treat the system by standard mathematical techniques (11). Thus we obtain:

$$P_{Zn} = a[1 - e^{-k_1 t} - k_1 e^{-k_1 t}/(k_2 - k_1) + k_1 e^{-k_2 t}/(k_2 - k_1)]$$

where P_{Zn} is the number of mating pairs formed by Zn^{2+}-treated males. For $k_1 = \infty$, corresponding to control males without Zn, the number of pairs would be given by:

$$P = a(1 - e^{-k_2 t})$$

Then,

$$P_{Zn}/P = [1 - e^{-k_1 t} - k_1 e^{-k_1 t}/(k_2 - k_1) + k_1 e^{-k_2 t}/(k_2 - k_1)]/(1 - e^{-k_2 t})$$

This ratio, P_{Zn}/P, is dependent primarily on the value of k_1 and insensitive both to the value of k_2 and to a, the highly variable mating efficiency obtained from day to day. Using a fixed k_2 (at 0.23 min^{-1}, half-time = 3 min) and different k_1 values, we have plotted three computed curves relating P_{Zn}/P and time in Fig. 3. It is seen that most of the ratios (N_t/N'_t) of recombinant counts fall between the upper and the lower curves, and are well fitted to the middle curve (k_1 = 3.46 min^{-1}, half-time = 12 sec) within experimental error. These results suggest two conclusions: (i) Zn^{2+} acts on the male, presumably on the tips of F pili; (ii) the effect of the male is reversible with a half time about 12 sec for males to recover after the Zn^{2+} has been removed.

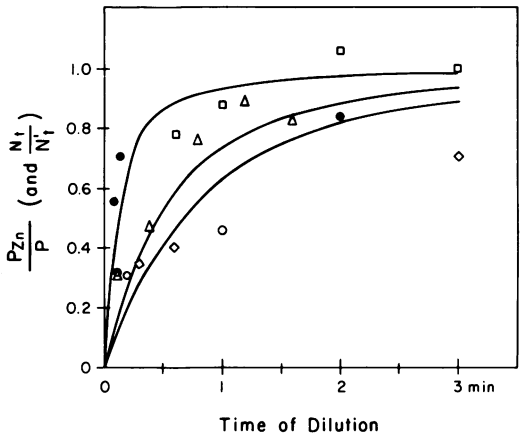

FIG. 3. *Kinetics of recovery of the fertility of males from Zn^{2+} treatment. See Materials and Methods for experimental procedures. Ordinate is the ratio of the number of mating pairs formed by Zn^{2+}-treated males, P_{Zn} (measured by the frequency of recombinants N_t), to those formed by untreated males, P (measured by the frequency of recombinants, N_t'), and the abscissa is the time of dilution. Symbols: ○, experiment 1; □, experiment 2; ◇, experiment 3; △, experiment 4; and ●, experiment 5. Solid curves are plotted by computation with k_2 = 0.23/min (half-time = 3 min) and k_1 = 13.86/min (half-time = 3 sec) for upper curve; k_1 = 3.46/min (half-time = 12 sec) for middle curve; and k_1 = 2.31/min (half-time = 18 sec) for lower curve.*

Converse experiments in which male bacteria were dumped onto female bacteria in 10^{-3} M Zn^{2+} yielded essentially the same numbers of recombinants as controls in which males were dumped onto female bacteria in broth (Table 3). These results indicate that any inhibitory effect 10^{-3} M Zn^{2+} might have on female receptor sites for pili is rapidly reversible with a half time of a few seconds after the Zn^{2+} has been diluted by the culture containing the male bacteria.

Effect of Zn^{2+} on chromosome mobilization. After mating pairs are formed, about 5 min is required for males to mobilize their chromosomes in preparation for the transfer of genetic material to the female (19). We have performed experiments like that of Fig. 4 to see if Zn^{2+} would slow mobilization and hence increase the times at which markers enter the females. It is seen that 10^{-3} M Zn^{2+} had no detectable effect on the times of entry of the markers *lac*$^+$ or *leu*$^+$. This indicates that Zn^{2+} did not delay mobilization nor did it slow the rate of chromosome transfer. However, it did decrease the number of recombinants formed as though it had disrupted some of the mating

TABLE 3. *Recovery of fertility after exposure of females to 10^{-3} M Zn^{2+} as measured by the efficiency of formation of Lac$^+$ recombinants*[a]

Expt no.	Broth control[b]	Broth + Zn^{2+}
1	0.044	0.090
2	0.020	0.029
3	0.010	0.010
4	0.025	0.190
5	0.100	0.130
6	0.010	0.010
7	0.010	0.012
8	0.550	0.430
9	0.260	0.290
10	0.260	0.210

[a] See text for experimental procedure. Seconds allowed for pair formation, 15; t value in Student's t test, 0.713; degrees of freedom, 9; probability, ~0.5.

[b] See footnote of Table 2 for the explanation of the variations in the frequency of recombination.

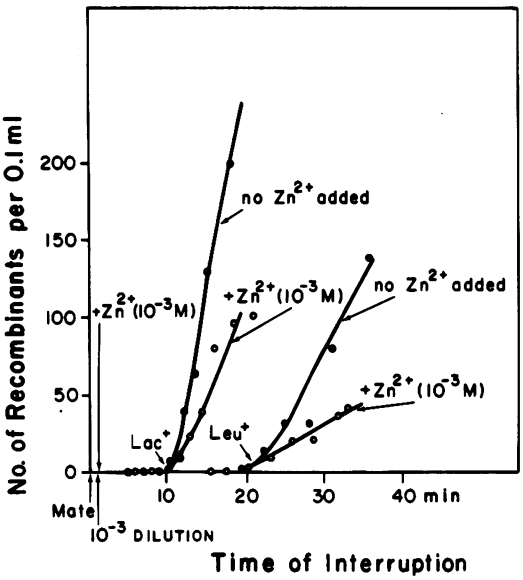

FIG. 4. *Effect of 10^{-3} M Zn^{2+} on entry times of the markers lac+ and leu+. Strains HfrW3011 and F- W1-3 were grown separately in broth and at time t = 0 mixed in equal proportions. At t = 1.5 min, some mating pairs had been allowed to form and the mixture was gently diluted 1:1,000 in 100 ml of broth. One sample of this diluted mixture was made 10^{-3} M in Zn^{2+}, whereas another sample served as a control. Periodically thereafter a 0.1-ml sample was added to 3 ml of soft agar which was then blended to interrupt mating and plated on plates suitable for detecting Lac+ and Leu+ recombinants. In the figure, the numbers of recombinants are plotted as ordinates against the times of blending for samples with (O) and without (●) added Zn^{2+}.*

pairs, had increased the rate of chromosome breakage during transfer, or proved lethal to some of the zygotes.

To minimize the possibility of lethality, both the males and females tested were adapted to Zn^{2+} by growth in 10^{-3} M Zn^{2+} (Fig. 5). Again the entry time for *lac+* was unaffected when Zn^{2+} was added at 6 min. Furthermore, the rise time (i.e., the time required for the number of recombinants to reach the plateau value) was unaffected, again indicating that neither mobilization nor rate of transfer was affected by Zn^{2+}.

In agreement with the results of Fig. 4, we note that even the preconditioned bacteria of Fig. 5 gave fewer recombinants in media containing Zn^{2+} than in controls. The ultimate number of recombinants formed was reduced to about 50% of the control when Zn^{2+} was added at t = 6 min and to about 90% when Zn^{2+} was added to t = 20 min. This could be due to: (i) Zn^{2+} disrupting some unstable mating pairs or (ii) Zn^{2+} increasing the rate of chromosomal breakage during transfer. The results of the next section show that the rate of chromosome breakage is not affected by Zn^{2+}.

Effect of Zn^{2+} on chromosome breakage. A number of workers have shown that during transfer there is a constant probability of chromosome breakage per unit length or time of transfer (17, 28) such that the number of recombinants for a gene at a distance x min from the original is given by

$$N(x) = N_0 A\, e^{-kx} \qquad (7)$$

Here N_0 is the number of minority parents in the mating mixture, A is a constant, and k is the gradient number (28). Thus, if Zn^{2+} were to increase the probability of chromosome breakage during transfer, it would increase the value of k. As shown in Table 4, 10^{-3} M Zn^{2+} had no apparent effect on k. We conclude that Zn^{2+} probably disrupts some of the mating pairs but that otherwise it has no detectable effect on the rate of chromosome breakage.

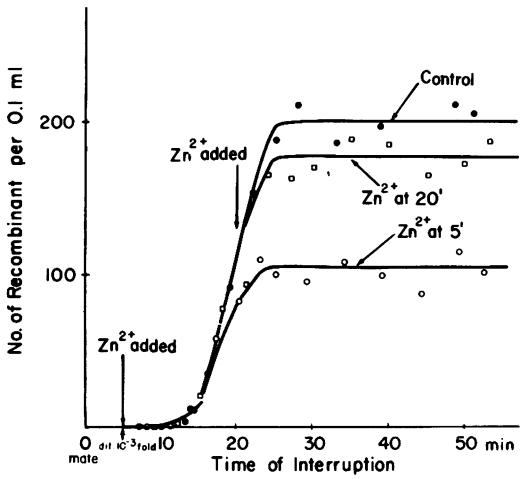

FIG. 5. *Effect of 10^{-3} M Zn^{2+} on entry time, on rise time, and on the ultimate number of Lac+ recombinants. This experiment is similar to that of Fig. 4 except that the bacteria were grown in broth containing 10^{-3} M Zn^{2+}. They were washed in broth and mixed at time t = 0. At time t = 5 min, the mixture was gently diluted 1:1,000 in broth (to prevent the formation of more mating pairs), and, at t = 6 min, the diluted mixture was divided into three flasks, one of which contained enough Zn^{2+} to make the sample contain 10^{-3} M Zn^{2+}. At t = 20 min, another sample was made 10^{-3} M Zn^{2+}. The third sample served as a control. At various times, 0.1 ml of each sample was added to 3 ml of soft agar, blended to interrupt mating, and plated for detection of Lac+ recombinants. Symbols: Zn^{2+} added at t = 6 min (O), at t = 20 min (□), and control (●).*

TABLE 4. *Effect of 10^{-3} M Zn^{2+} on chromosome breakage during transfer*[a]

Expt no.	Broth			Broth + 10^{-3} M Zn^{2+}		
	N (x)/N_o		k_o	N (x)/N_o		k_{Zn}
	Lac$^+$	Leu$^+$		Lac$^+$	Leu$^+$	
1	0.344	0.147	0.094	0.111	0.043	0.104
2	0.208	0.089	0.094	0.084	0.038	0.089
3	0.212	0.089	0.097	0.073	0.030	0.100
4	0.178	0.073	0.100	0.064	0.026	0.100
Avg			0.096			0.098

[a] Male and female bacteria were mixed at time t = 0; after 3 min, one sample was gently diluted 1:1,000 in broth and another diluted 1:1,000 in broth containing 10^{-3} M Zn^{2+}. Chromosome transfer was allowed to continue in the diluted mixtures for 90 min, when samples were blended to interrupt mating and Lac$^+$ and Leu$^+$ recombinants were scored. Values of the gradient number, k, were calculated from equation 7 using $x = 10$ and $x = 19$ min for the entry times of *lac*$^+$ and *leu*$^+$. Student's t test applied to the paired values of k in the four experiments showed that Zn^{2+} had no detectable effect on chromosome breakage. Value of t in Student's t test, 0.69; degrees of freedom, 3; probability, 0.6.

Effect of Zn^{2+} on integration. The frequency of recombinant formation would be less than that of the control if Zn^{2+} selectively destroys recombinants or somehow interferes with the process of normal integration. This was tested by incubating exconjugants in broth with and without Zn^{2+} and determining the number of recombinants as a function of time, as described above.

The results (Fig. 6) showed that the number of recombinants was the same in the samples incubated both with and without added Zn^{2+}. Moreover, the number stayed constant, suggesting that Zn^{2+} is at least not preventing the eventual integration of the transferred chromosome. However, Zn^{2+} did lengthen the time at which the number of recombinant colonies increased from 110 min in the control culture to 170 min after mixing in the Zn^{2+}-treated culture. The fact that a corresponding delay is also seen in the growth of F^- cells transferred to a Zn^{2+}-containing medium suggests that the delay in growth of recombinants is simply caused by an effect of Zn^{2+} on growth and division. We conclude, therefore, that Zn^{2+} did not block integration nor selectively suppress (or kill) recombinants but that it did inhibit cell division.

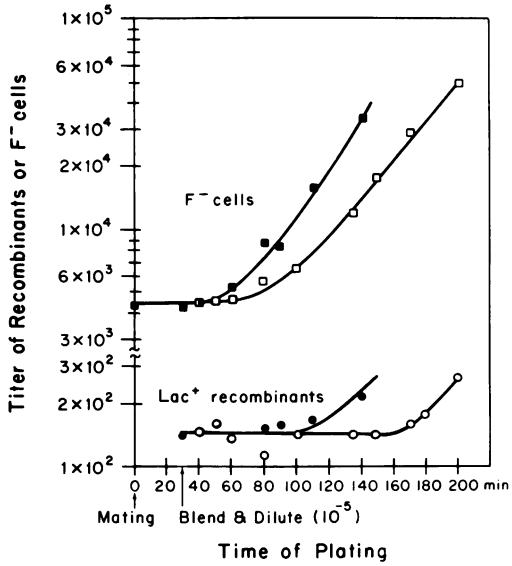

FIG. 6. *Effect of 10^{-3} M Zn^{2+} on recombination and segregation. Hfr cells grown in broth and F^- cells grown in 10^{-3} M Zn^{2+} were washed, resuspended in broth, and, at time t = 0, mixed in equal proportions. Mating was allowed to proceed in broth for 30 min when the mixture was diluted 10^{-5} in broth and mating interrupted by blending. The diluted preparation was then divided into two samples; one served as a control, whereas the other was made 10^{-3} M in Zn^{2+}. At various times thereafter, samples were plated to count Lac$^+$ recombinants. The squares represent the titer of F^- cells with (□) and without (■) added Zn^{2+}. Circles represent the titer of Lac$^+$ recombinants with (○) and without (●) added Zn^{2+}.*

DISCUSSION

Our results show that Zn^{2+} at 10^{-3} M blocks the first step in mating: the formation of mating pairs. To see if Zn^{2+} affects males (F pili) or females (receptor spots), we exposed them separately to Zn^{2+} for brief periods and measured their fertility at various times after removing the Zn^{2+}. The results indicate that if the female could possibly have been rendered infertile by Zn^{2+}, the recovery of fertility must have been so rapid that its effect became negligible within 15 sec. On the other hand, the sterilizing effect of 10^{-3} M Zn^{2+} on the male persists with a half-time of about 12 sec after Zn^{2+} is removed.

From our data there are two ways to calculate an approximate dissociation constant, K_D, for the reaction between the Zn^{2+}-sensitive spot(s) and free Zn^{2+}. The first involves a calculation of the free Zn^{2+} in the Zn^{2+}-enriched medium that effectively inhibits conjugation. The total concentration of Zn^{2+} in such media is 10^{-3} M, but the free Zn^{2+} is much lower than this because the amino acids in

broth bind Zn^{2+} with an average binding constant of 3×10^{-5} mole/liter (23). Our nutrient broth contains about 0.05 mole of free amino acid groups per liter as measured by the ninhydrin reaction. From this, the free Zn^{2+} concentration can be calculated to be only 6×10^{-7} M when the total Zn^{2+} concentration is 10^{-3} M. The K_D of the pili tips for free Zn^{2+} must therefore be of the order of 10^{-6} to 10^{-8} mole/liter.

The second method estimates the binding constants from the rate with which the reaction is reversed on removing excess Zn^{2+}:

$$K_D = k_{off}/k_{on}$$

where k_{off} is the rate of release of Zn^{2+} and k_{on} is the rate of addition of Zn^{2+} to the sensitive complex. We estimated the half-time for dissociation of the pili-Zn^{2+} complex to be 12 sec which gives a value of $k_{off} \approx 0.058$/sec. On the other hand k_{on} for uncomplicated reactions of Zn^{2+} to form metal complexes is about 10^6 liters per mole per sec (13). Thus K_D by this method is about 5.8×10^{-8} moles/liter. Both methods of calculation therefore indicate that the sensitive sites on F pili have high association constants for free Zn^{2+}.

The reversibility of the effect of Zn^{2+} on the male is to be contrasted with the essentially irreversible effect of metaperiodate ($NaIO_4$) on the fertility of males discovered by Sneath and Lederberg (25). Subsequently, Dettori et al. (12) showed that $NaIO_4$ also inhibits the adsorption of the male-specific spherical RNA phage μ_2 to male bacteria. Since these phages are known to attach to the sides of F pili (6, 7), $NaIO_4$ would appear to inactivate the μ_2 receptors on their sides. Recently C. E. Raizen (Ph.D. thesis, Univ. of Wisconsin, 1967) extended the work of Dettori et al. and showed that $NaIO_4$ inhibits the adsorption of both types of male-specific phages—the thread-shaped DNA phage to the tips and the spherical RNA phages to the sides of F pili. The work of Raizen suggests that $NaIO_4$ destroys phage receptor sites both on the sides and on the tips of F pili.

$NaIO_4$ would be expected to alter F pili by an irreversible oxidation; indeed, males remained infertile for 30 min after the $NaIO_4$ had been removed and required as much as 120 min to recover their normal fertility (25). Presumably, the recovery is due to the regrowth and conditioning of new pili. Regrowth is relatively fast. According to Brinton (4), after pili have been mechanically removed from males in a blendor, only 6 min is required for 50% of pili structures to be reformed. However, the much longer time of 60 min is required for the recovery of only 13% of the donor ability of unblended cells (4) as though new pili had to be conditioned in some way to be fully effective as mating organelles. In contrast, Zn^{2+} exerts no such permanent damage but can simply be washed away in a minute or less as it can be from chelating agents. However, the effect of Zn^{2+} is relatively specific, for other divalent cations such as Mg^{2+} or Ca^{2+} have negligible effects on fertility.

We have found no evidence that Zn^{2+} affects later stages in the mating process: chromosome mobilization in the male, its transfer to the female, or its integration within the zygote. The interrupted mating experiments of Fig. 5 show that the addition of Zn^{2+} to mating pairs that had formed 6 min before reduces the number of recombinants by a factor of two. This fact together with the observation that the earliest time of marker entry was unaffected (showing that mobilization was unaffected) and that Zn^{2+} had no effect on the rate or gradient of transfer (Table 4) suggest that there are at least two stages in the formation of mating pairs: an early stage that can be disrupted by the addition of Zn^{2+} and a later stage that is unaffected by Zn^{2+}. The half-life of the sensitive stage is about 5 min so that a detectable proportion of these Zn^{2+}-sensitive pairs still persists for as long as 20 min after mating pairs have been formed (Fig. 5).

Zn^{2+} also blocks the adsorption of the thread-shaped male-specific DNA phage, M13, to the tips of F pili (27). This can be seen directly in the electron microscope (Ou, *unpublished data*). It would seem from all our observations that Zn^{2+} acts on the tips of F pili to block the adsorption of both M13-type phages and receptor sites on female bacteria.

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