Some Effects of Nalidixic Acid on Conjugation in *Escherichia coli* K-12

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The role of deoxyribonucleic acid (DNA) synthesis in the *Escherichia coli* conjugation system has been studied using nalidixic acid (NAL) to specifically inhibit DNA synthesis in matings between reciprocal combinations of male (Hfr) and female (F -) mutants resistant and sensitive to NAL; the physiological action of NAL on the strains utilized was also studied. Matings between combinations of mutants resistant (NAL +) and sensitive (NAL -) to NAL allow various parental functions to be established: pair formation studies show that the female cells are responsible for the slight decrease in pair formation when NAL is present in Hfr(Nal +) × F - (Nal -) matings. Preformed mating pairs are stable in the presence of NAL. In matings between Hfr(Nal +) and F - (Nal -), the transfer gradient constant increases linearly with low NAL concentration (0.1 to 0.6 µg of NAL per ml). Higher concentrations of NAL (5 µg/ml) act on Nal + males to rapidly stop chromosome transfer; under these conditions, however, DNA degradation is unmeasurable as determined from single-strand nicking in the male cells. This result is consistent with a model for chromosome transfer which requires DNA synthesis in the male cell. Inhibition of DNA synthesis (by 85%) in the female has no effect on conjugal chromosome transfer. High concentrations of NAL (> 20 µg/ml) produce slight inhibition in chromosome transfer for the Hfr(Nal +) × F - (Nal -) mating tested; this effect is probably caused by action of NAL on the male. The inhibition of chromosomal transfer by NAL appears to be irreversible in the normal sense. A pulse of NAL, applied during transfer, immediately stops the transfer which is in progress. On removal of the NAL block, the temporal appearance of recombinants is consistent with the idea that a new round of transfer has commenced from the sex factor location on the male chromosome.

The process of bacterial conjugation consists of several consecutive steps [see, for example, Wood and Walmsley (36)]; (i) a pairing between a donor cell (male) and a recipient cell (female) which leads to an effective union; (ii) the polarized transfer of a portion of a donor chromosome into the recipient cell; and (iii) the integration of part of the genetic information thereby transferred with that of the recipient parent into a recombinant chromosome which is subsequently segregated from those of the female parent. The area of bacterial conjugation has been reviewed recently by Curtiss (12) and Adelberg and Pittard (2).

Many recent experiments have dealt with the mechanism of chromosome transfer. There is general agreement that deoxyribonucleic acid (DNA) synthesis is necessary for some aspects of transfer but disagreement about the transfer mechanism and even about the relative roles of male and female parents. Although the majority of results are consistent with the model proposed by Jacob, Brenner, and Cuzin (25), most are inconclusive either because the system utilized was not thoroughly characterized or because the magnitudes of the effects were too small. The clearest conclusions regarding the role of DNA replication are from the results of Gross and Caro (21) and Ptashne (32); these establish that DNA is replicated during transfer and that one newly labeled strand (labeled during transfer) appears in the female.

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It seems likely now that the transferred DNA enters the female as a single strand. Strong evidence for this comes from the experiments of Cohen et al. (11), from Rupp and Ihler (33) and especially from Ohki and Tomizawa (28). These results are consistent with those of Gross and Caro (21) and Ptashne (32) if the transferred single strand is rendered double stranded either during or after mating.

The recent discovery of nalidixic acid (NAL; 1-ethyl-7-methyl-4-oxo-1,8-naphthyridine-3-carboxylic acid), reported to specifically inhibit DNA synthesis (20), appeared to provide a useful way to explore the role of DNA synthesis in conjugation. Holom and Pritchard (23) first used NAL to inhibit DNA synthesis during conjugation. They observed a rapid inhibition of recombinant production when NAL was added during transfer and concluded that the inhibition was reversible; their results did not specify which parent(s) required DNA synthesis.

Barbour (3) extended the usefulness of NAL by isolating mutants resistant to the drug: he then studied F' Lac + transfer (by β-galactosidase assay) in the presence of NAL by using reciprocal combinations of sensitive and resistant male and female strains. Whenever the male cell was sensitive, no transfer occurred; when the male was resistant and the female sensitive, normal transfer took place. Fisher and Fisher (17) and Bresler et al. (8) have recently studied the effect of NAL on matings between Hfr and F- bacteria resistant or sensitive to this agent; recombinant production was greatly affected only with sensitive males. The above studies have dealt with the overall process of recombinant production and have not examined the effect of NAL on the individual steps of conjugation.

In the present study matings between donor and recipient bacteria which are resistant (Nal') or sensitive (NAL') to nalidixic acid are carried out under conditions which allow determinations to be made of the effect of this agent on the various steps of conjugation.

**MATERIALS AND METHODS**

**Bacterial and viral strains.** The *Escherichia coli* K-12 strains used in this research are listed in Table 1. Nomenclature follows the suggestions of Demerec (16) and Taylor and Trotter (34).

A mutant clear-plaque phase, λ<sub>imm</sub>, was utilized in zygotogenic induction experiments. This mutant was isolated from strain DF105(λ<sup>+</sup>) sent to our laboratory by D. Freifelder. Stocks of λ<sub>imm</sub> were grown in an indicator strain (C6000) and purified, yielding about 2 × 10<sup>10</sup> phage/ml. The isolation and characterization of Nal<sup>+</sup> strains is described elsewhere (22). Lysogenic Nal<sup>+</sup> strains were always isolated from Nal<sup>-</sup> strains since lysogenic Nal<sup>+</sup> strains are induced by NAL (18).

**Media.** Cells were grown and mated either in broth or synthetic medium. Broth contained 5 g of NaCl, 5 g of proline, and 3 g of nutrient broth (Difco) in 1 liter of distilled water. Synthetic medium was composed of an M-9 salts stock (1) supplemented with tryptophan, arginine, and vitamins to the following concentrations (in μg/ml): threonine, 40; leucine, 20; tryptophan, 10; proline, 20; histidine, 20; thiamine, 1; glucose, 2,000; adenine, 20 and thymine, 50.

Phenotypic selection was performed in synthetic medium lacking one or more of these metabolites or supplemented with a growth inhibitor. Streptomycin, at 100 μg/ml, was the most common male counterselective chemical. These media were gelled with 1.5% agar to form assay plates for parental and recombinant genotypes. Topping agar was 0.75% agar, M-9 solution stored in 3-ml portions at 50°C.

**NAL preparation.** NAL was generously supplied as a pure powder from the Sterling-Winthrop Research Foundation, Rensselaer, N.Y. NAL stock solutions (200 μg/ml) were routinely prepared by dissolving NAL in M-9 or broth and autoclaving for 15 min. When higher concentrations of NAL were needed, NAL was dissolved in 0.1 N NaOH to form the sodium salt. The biological activity of NAL was not affected by autoclaving or preparation as sodium nalidixate.

**Mating methods.** Bacteria were normally grown overnight at 37°C in broth with agitation to stationary phase. Matings followed different protocols depending on the experiment. Standard 90-min transfer gradients were obtained by diluting (1:100) the overnight culture in fresh broth and growing the cells with agitation to about 3 × 10<sup>8</sup> cells/ml (exponential growth). When maximum efficiency of pair formation was desired, the male culture was allowed to stand without agitation for 30 to 60 min before mating. This procedure results in heavy accumulation of male F pil and good pair formation (9, 13). Male cells were mixed with female cells

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<sup>*Unlisted genetic characters are assumed to be wild type. Abbreviations for (i) nutritional requirements: thr, threonine; leu, leucine; his, histidine; B<sup>+</sup>, thiamine; trp, tryptophan; arg, arginine; (ii) ability to utilize sugars: lac, lactose; xy, xylose; mtl, maltose; man, mannose; gal, galactose; (iii) resistance to agents: str, streptomycin; nal, nalidixic acid; (iv) λ, lysogenic for phase λ.</sup>
in the ratio 1:10 and mated with gentle agitation for the desired time—usually 90 min; the mating mixture was diluted and plated without blending on selective plates. Plate incubation time at 37°C varied from 18 hr for rich media to 48 hr for synthetic plates.

Chromosome transfer kinetics were examined by means of interrupted-mating experiments. Cells were grown in broth as described above and mated in male to female ratios between 1:1 and 1:10, depending on the experiment. To obtain the earliest gene entry time, the mating mixture was gently diluted five- or tenfold into fresh broth after 5 min. The dilution, which prevents a decreased transfer of distal markers, has only a slight effect on subsequent pair formation and gives reproducible entry curves. Portions of the mating suspension were pipetted into 3 ml of topping agar and blended for 5 sec in the Low-Wood apparatus (27), and the blended mixture was poured directly on selective plates. This technique allowed rapid sampling (every 30 sec) and efficient blending of the mating mixture.

Pulse matings (1,000-fold dilution after a 5-min mating) followed the method of de Haan and Gross (14). Plateaus for entry curves were obtained only under conditions when female growth was greatly diminished during the mating. Low female growth was accomplished by growing male and female cells to 2 × 10^9/ml in minimal media supplemented with 10% broth. After pulse mating, the mixture was diluted 10^-4 into warm M-9 supplemented with glucose.

**Measurement of pair formation.** Pair formation kinetics was studied by use of a method developed by R. Walmsley (unpublished data). When cells of opposite mating types are mixed together, the total bacterial count of the suspension as measured by a Coulter Counter decreases with time to asymptotic value which is characteristic of the number of minority parents in the mating mixture; this method allows measurement of the fraction of mechanically paired bacteria.

Female and male bacteria were mated in a ratio of 1:1 and separate portions of male and female bacteria were incubated in parallel during the experiment. Under conditions of 1:1 mating, the number of pairs at a given time is P = M/2 + F/2 − MM, where M, F, and MM are the Coulter counts in the male, female, and mating mixture tubes, respectively.

**Zygotice induction.** Genetic transfer under conditions requiring little or no integration of the transferred material was measured by zygotice induction of prophage λ in interrupted-mating experiments. Lysogenic male cells in exponential growth were washed by filtration or centrifugation to reduce the titer of free phage and resuspended in fresh medium. Proximal amino acid markers were assayed in the normal way. Entry of λ was measured by blending 0.1 ml of the mating mixture with 0.5 ml of C600 indicator cells in the soft-agar tubes and plating onto broth + streptomycin plates. Plaques were counted after 1 day of incubation.

**DNA synthesis.** DNA synthesis was measured by the uptake of [14C]-thymidine in the presence of 250 µg of deoxyadenosine per ml (5). DNA degradation was computed from the loss of acid insoluble counts from cultures grown for at least three generations in [14C]-thymidine.

**Alkaline denaturation of DNA and sucrose gradient analysis.** The techniques described by Kozinski, Kozinski, and James (25) were utilized. Bacterial pellets were washed once in 0.15 M NaCl, 0.015 M ethylenediaminetetraacetic acid (EDTA) buffer (pH 8.0) and then resuspended in the same medium. To 0.1 ml of resuspended bacteria was added 0.1 ml of PM medium [0.05 M tris(hydroxymethyl)aminomethane (pH 8.0), 0.05 M NaCl, 0.01 M EDTA, and 100 µg lysozyme per ml]. The mixture was kept on ice for 10 min, and then Triton X-100 was added to a final concentration of 1% for 5 min. Fifty µl of 1 M KOH was added, and the samples were incubated for 20 min at 37°C. Labeled [32P] T1 phage was then added so that the total 32P and 14C counts were approximately equal, and incubation was continued for 5 min at 37°C. This preparation was then layered on neutral sucrose gradients and centrifuged for 150 min at 28,000 rev/min in a Beckman SW 50.1 rotor. This procedure allows recovery of single-strand molecules which are three to five times as long as T4 phage single-strand molecules. Recovery was calculated by comparison of the input with the sum of the counts recovered in the fractions.

**RESULTS**

**Physiological experiments.** Several experiments were carried out to determine the physiological responses of the strains used to NAL. In an earlier paper (22), we reported that three classes of mutants can be identified by their sensitivities to NAL. Wild-type cells (Nal*) are sensitive to 2 µg of NAL per ml, NalB mutants are resistant to 4 µg but sensitive to 10 µg of NAL per ml, and NalA* mutants are resistant to 40 µg, or more, of NAL per ml. The resistant mutants (NalA*) used in the present work are sensitive to 60 µg of NAL per ml. The merodiploid nalA*/nalA* is phenotypically NalA+ (22).

In HfrH (Nal*), DNA synthesis as measured by [14C] uptake showed 85% inhibition by 5 µg of NAL per ml and 90% by 20 µg/ml with 60 min of incubation; in the corresponding NalA* strain, these concentrations give 0 and 10% inhibition, respectively. Protein synthesis (measured by [14C]-leucine uptake) and RNA synthesis (measured by [14C]-uridine uptake) were unaffected during 60 min of incubation with 5 µg of NAL per ml. These results are consistent with those of Goss et al. (19, 20) and Winshell (Ph.D. Dissertation, Columbia University, 1967).

Concomitant protein synthesis was found necessary for NAL-induced lethality, although, as reported by Deitz et al. (15), protein synthesis was not necessary for NAL-induced DNA synthesis inhibition. In addition, prior inhibition of DNA synthesis by hydroxyurea did not reduce NAL-induced killing in contrast with the results obtained with this treatment by Winshell with *E. coli* B (Winshell, Ph.D. Dissertation).

Degradation of DNA after prolonged treatment with NAL has been reported for *E. coli*.
15TAU and *E. coli* 198 by Cook et al. (10), who warn "against the uncritical use of NAL... in genetic studies." DNA is also degraded to a slight extent in the strains used here. After 60 min of incubation, HfrH DNA is not detectably degraded by 5 μg of NAL per ml but shows 6% degradation by 20 μg/ml; PA309 DNA is degraded 3 and 11%, respectively, by these same concentrations. To determine whether this degradation was due to extensive chromosomal nicking, an exponentially growing culture of HfrH was exposed to various concentrations of NAL; DNA from these cells was subjected to alkaline denaturation and sucrose gradient analysis. Data from four sucrose gradients are presented in Fig. 1. The control shows that the alkali-denatured DNA isolated from untreated cells has a molecular-size distribution expected for large DNA molecules. The DNA strands isolated after treatment with NAL (Fig. 1b, c, d) have a size distribution similar to that of the control culture—that is, with DNA molecules three to five times as long as T4 molecules. Since T4 DNA contains approximately \(2 \times 10^8\) nucleotide bases, these single-strand molecules probably contain \(3 \times 10^5\) to \(5 \times 10^5\) nucleotides.

**Effects of NAL on conjugating bacteria: mating pair formation between NAL-treated cells.** Measurement of pair formation utilizing a Coulter Counter is more specific than the ordinary method which measures formation of only those pairs which eventually produce clones of recombinant bacteria. This technique is especially applicable in experiments in which pair formation is being studied under conditions which might produce irreversible inhibition of chromosome transfer.

When NAL, either at 5 or 20 μg/ml, is added to a mating (1:1) between Nal<sup>+</sup> male and Nal<sup>−</sup> female cells, no inhibition of pair formation is detectable (Fig. 2A). However, when the male is Nal<sup>+</sup> and the female Nal<sup>+</sup>, 20 μg of NAL per ml causes approximately 50% inhibition of pair formation (Fig. 2B). If NAL (20 μg/ml) is added at 30 min when pair formation is essentially complete, there is no reduction in the number of pairs even after 40 min of exposure to either situation. These results are consistent with those of Hollom and Pritchard (23), who found a similar inhibition in pair formation between Nal<sup>−</sup> males and females but could not identify the responsible parents. These results suggest that some modification of the cell wall may occur in sensitive female strains, which leads to a loss of pair formation receptor sites, perhaps sites recognized by the F pilus.

**Interrupted-mating experiments.** The first mating, in which NAL was added during conjugation, was performed to check the observation that NAL acts mainly on the male partner when it stops recombinant production (8). Strains HfrH (Nal<sup>−</sup>) and F<sup>−</sup>MH1000 (Nal<sup>+</sup>) were mated in the ratio 1:10 for 5 min and diluted 10-fold into three flasks. Portions (0.1 ml) were blended at frequent intervals and plated for leucine (Leu), tryptophan (Trp), and histidine (His) recombinants; the entry times at 37°C for these markers were known from previous experience to be: leu,

![Fig. 1. Neutral sucrose gradient analysis of alkali-denatured DNA isolated after in vivo treatment with nalidixic acid.](http://jb.asm.org/download/105/1971/49)
8 min; trp, 33 min; and his, 52 min. NAL (20 μg/ml) was added to one flask at 9.5 min (B) and to another at 58 min (C) after mating; the third flask (A) served as a control. NAL (2 μg) was carried over onto the plates in the 3 ml of topping agar; however, this concentration of NAL has no effect on recombinant production.

Each of the control entry curves for this experiment (Fig. 3) shows a normal, monotonic rise starting at the marker entry time. (Note that this scale displays only the beginning of the entry curves.) In contrast, when NAL is added at 9 min (flask B), the leu entry curve flattens out within 1 min and remains constant for at least 60 min; no recombinants appear for the distal markers, trp (solid circles) or his (solid squares). The entry curves for flask C are also normal until NAL is added at 58 min; after this addition, the His entry curve immediately flattens out at the level of recombinants which had entered at 58 min. The minimum level of NAL necessary to rapidly stop chromosome transfer under these conditions (broth-grown cells, mating with gentle agitation) was determined to be 5 μg of NAL per ml. NAL (1 μg/ml) stops transfer gradually when it is added at the gene entry time and allows further transfer of approximately 25% as many genes as the control. Note that 5 μg of NAL causes no measurable DNA degradation in HfrH.

One could argue that, in this experiment, the male chromosome continued to be transferred in the presence of NAL but was modified in some way which prevented its recombination after entering the resistant female cells. Zygotic induction, which requires no recombination of the transferred prophage, allowed confirmation that the apparent halt in transfer was not caused by a process which inhibited recombination. Strains Hfr MH2 [Hfr H (Naλ+)] and MH1005 [P678 (Naλ−)] were grown to mid-exponential phase in broth. MH2 was centrifuged and washed twice with fresh broth, to reduce the number of free λ phage in solution, and resuspended in broth without agitation for 5 min before mating. MH2 and MH1005 were then mated in the ratio 1:10 and, after 5 min, diluted 10−8 into three flasks of prewarmed male medium. Portions were blended at frequent intervals and plated for Leu recombinants or for assay of λ infective centers. (A 0.5-ml amount of C600 (str') indicator strain was blended with the λ assays to provide the lawn for plaque development on broth + Str plates.) A 10−4 dilution of MH2 (in M-9 + glucose medium) was incubated, and portions (0.05 ml) were periodically mixed with 0.5 ml of C600 and plated on broth + Str plates as controls on the number of free λ and of the MH2 which mated with the indicator strain. The control resulted in a constant level of 80 plaques per 0.05 ml, and the following results were corrected for this background. NAL (20 μg/ml) was added to one of the flasks (B) at 12 min after mating and to another (C) at 33 min. NAL at 20 μg/ml was used rather than a lower concentration (5 μg/ml would have been sufficient) since 20 μg of NAL per ml causes induction of largely noninfectious phage.

Entry of the Leu control occurs at 10 min under these conditions (Fig. 4), and the prophage (λ) enters at 30 min after mating. Dilution of the mating mixture 10−2 into male medium results in a decline after maximum entry of leu which had been attributed to chromosome withdrawal by de Haan and Gross (14). The Leu recombinant level continues to decrease after λ entry, presumably because those Leu recombinants which subsequently inherit λ prophage (about 30%) are lysed.

Note also that the maximum number of Leu re-

FIG. 2. Effect of NAL on pair formation: (a) HfrH(Naλ+) x MH1000(Naλ+) (b) Hfr-MH1(Naλ+) x PA309(Naλ+).

FIG. 3. Halt in recombinant production when NAL is added during transfer from HfrH(Naλ+) to MH1000(Naλ+). The cells were mated 1:10 and diluted 10-fold at 5 min into three separate flasks. Samples were blended and tested for leu, trp, and his entry. NAL (20 μg/ml) was added to flask B at 9 min and to flask C at 58 min after mating.
combinants is about one-third the number of \( \lambda \) plaques, although \( \lambda \) is transferred 20 min later than \( \text{leu} \). This suggests that only about 12% of the \( \text{leu} \) genes which enter females result in recombinants in this case.

When NAL is added to flask B at 12 min, the \( \text{leu} \) entry curve flattens out and remains constant. The \( \lambda \) assay for flask B (Fig. 4, dashed curve) shows, after background correction, a slight increase (less than 10% of the control) which is attributed to induction of a few viable \( \lambda \) from the male cells. Similarly, when NAL is added at 33 min (Fig. 4, half-shaded squares), the entry curve flattens out rapidly and remains constant for at least 40 min. The equivalent inhibition of \( \text{leu} \) and \( \lambda \) assays in this experiment indicates that processes involved with recombination are unrelated to the halt in transfer.

Next, the role of the female was studied in a similar manner, by using male \( \text{NaI}^+ \) and female \( \text{NaI}^- \) cells. Preliminary experiments resulted in little transfer inhibition when the female was \( \text{NaI}^+ \), so it appeared desirable to study complete entry curves to determine whether any inhibition could be detected. \( \text{Hfr MH}2(\lambda^+\text{NaI}^+) \times \text{MH}1005(\lambda^-\text{NaI}^-) \). At 5 min, the mating mixture was diluted 10-fold into three flasks. NAL (20 \( \mu \)g/ml) was added to one (B) at 12 min and to another (C) at 33 min; the third (A) served as a control. Portions were blended for \( \text{leu} \) and \( \lambda \) entry.

The control curves for \( \text{leu} \), \( \text{Trp} \), and \( \text{His} \) for this experiment (Fig. 5) deserve comment, since it may be puzzling that they do not each flatten out into a defined "textbook" plateau. In my experience, when matings are carried out in broth and diluted 1,000-fold into fresh broth, plateaus are never obtained; plateaus are obtained with starved female cells.

For the cultures treated with NAL (curves B and C in Fig. 5), the entry curves are almost identical to the control for most of the entry time. In culture C (5 \( \mu \)g/ml) female lethality began to occur at 45 min, and the entry curves at later times are corrected for female survival. For culture A, the females grew exponentially with a doubling time of 40 min; the female titer in flask B remained constant during the experiment.

The lack of chromosome transfer inhibition by NAL in this experiment is clear and is especially striking when compared to the immediate halt caused by 5 \( \mu \)g of NAL per ml in \( \text{NaI}^+ \) males. At about 10 min after lethality begins in culture C (5 \( \mu \)g of NAL per ml), all three entry curves become depressed compared to the control. Since these curves have been corrected for female lethality, they reflect an added interference with recombinant production after the onset of lethality. Such a decrease is reasonable, since approximately 3% of the female DNA is degraded after 70 min of exposure to 5 \( \mu \)g of NAL per ml. The extra decrease may be caused by degradation of some of the transferred male DNA or by interference with the recombination process—perhaps a repair step—or both.

Transfer gradient experiments. In the previous interrupted-mating experiments, NAL was added only after pair formation and mobilization for transfer had occurred, and plating out effectively reduced the NAL concentration to a noneffective level on the plates where most recombination occurs. Therefore the effects of NAL were only on the transfer process. The overall phenomenol-
ogy of conjugation can be studied by mating in the presence of NAL for a time adequate to allow completion of transfer of several male markers. The mating mixture is then diluted and plated; the number of viable recombinants \( N(x) \) for markers at distance \( x \) from the transfer origin are counted. Since, experimentally, \( N(x)/N_0 = Ae^{-kt} \) (see 36), the action of NAL on \( A \) (the extrapolate number) and \( k \) (the gradient constant) can be determined.

For 90-min matings between Hfr H (Nal\(^*\)) and MH1000 (Nal\(^*\)) bacteria, the gradient constant \( k \) is greatly increased by low levels of NAL (Fig. 6A); \( k \) is increased threefold by mating in the presence of 0.6 \( \mu \)g of NAL per ml. The extrapolate number, \( A \), however, is independent of the concentration of NAL. Note that \( A \) is measured by extrapolating the gradient plots back to the "origin of transfer," which is at about 5 min after mating since approximately 5 min is required for chromosome mobilization after mating-pair formation (2). We conclude that for Hfr H \( \times \) MH1000, the processes of pair formation and mobilization (measured by \( A \)) are independent of low-level concentrations of NAL, but that NAL strongly inhibits transfer.

When NAL is added during matings between MH1 (Nal\(^*\)) and PA309 (Nal\(^*\)), there is little change in either \( k \) or \( A \) (Fig. 6B). The data for 5 \( \mu \)g of NAL per ml have been corrected for female survival (50%) at 90 min. Note that much higher concentrations of NAL were used in this experiment than in the previous one; 5 \( \mu \)g of NAL per ml causes only a 40% increase in \( k \) when the female is Nal\(^*\). The major conclusion is that in sensitive recipients, pair formation, mobilization, and transfer are inappreciably affected by 1 or 5 \( \mu \)g of NAL per ml.

In several early experiments between MH1 (Nal\(^*\)) and PA309 (Nal\(^*\)), a significant depression in recombinants for distal markers (trp, his) was observed when high (20 to 40 \( \mu \)g of NAL per ml) concentrations of NAL were added to the mating mixture. Since these concentrations of NAL have little, if any, immediate effect on recombination processes in sensitive females, the depression appeared to be caused by transfer inhibition in the sensitive female at high NAL concentrations. To pursue this matter, comparable matings were carried out between Nal\(^*\) male (MH1) and Nal\(^*\) female (MH1000) cells in the presence of 20 and 40 \( \mu \)g of NAL per ml; the entry curves were depressed to the same degree as for matings with Nal\(^*\) females. Therefore it appears that the transfer inhibition results from processes in the male.

To quantitate this phenomenon, gradient experiments were carried out for Nal\(^*\) male – Nal\(^*\) female and Nal\(^*\) male – Nal\(^*\) female matings by using high concentrations of NAL. Results from several such experiments are shown in Fig. 7. Values of \( k \) for control matings (no NAL) vary somewhat in different experiments. Therefore the relative gradient constant, \( k(NAL)/k(control) \), is a more pertinent parameter for comparison of different experiments. The relative sensitivities for Nal\(^*\) male \( \times \) Nal\(^*\) female and Nal\(^*\) male \( \times \) Nal\(^*\) female matings are almost identical (Fig. 7); 40 \( \mu \)g of NAL per ml causes a 100% increase in \( k \). For comparison, the relative sensitivities for matings involving Nal\(^*\) males are plotted in Fig. 7 (note the expanded scale for NAL concentration; \( k \) (male Nal\(^*\))/\( k \) (male Nal\(^*\)) \( \approx \) 25.

The interpretation—that processes in the Nal\(^*\) male MH1 cause this transfer inhibition—is reasonable since DNA synthesis in MH1 is inhibited about 10% at 20 \( \mu \)g of NAL per ml and 20% at 40 \( \mu \)g of NAL per ml as discussed earlier.

Irreversibility of chromosome transfer by NAL: induction of a new round of chromosome transfer after exposure to NAL. Several preliminary interrupted-mating experiments, in which NAL was added to halt leu transfer (as in Fig. 3) in matings between Hfr H (Nal\(^*\)) and MH1000 (Nal\(^*\)), showed that, after dilution of NAL, leu transfer resumed after approximately an 8-min delay; thus the inhibition appeared reversible. Since, however, 8 min is also the normal entry time in this system, this question was studied further by asking whether both trp and the distal marker, his, would be transferred reversibly (with an 8-min lag) after a pulse of NAL. Hfr H and MH1000 were mated in the ratio 1:1 and diluted 10-fold into broth A' (see Fig. 8); portions

![Fig. 6. Gradient plots for (a) HfrH(Nal\(^*\)) \( \times \) MH1000(Nal\(^*\)) and (b) MH1(Nal\(^*\)) \( \times \) PA309(Nal\(^*\)). Ninety-minute matings were assayed for Leu, Trp, and His recombinants. Note the higher NAL concentrations in part b.](http://jb.asm.org/Downloaded from jib.asm.org on October 24, 2017 by guest)
(0.1 ml) were blended for control entry of *leu*. At 25 min, flask A' was split into flasks A and B; B was supplemented with NAL (to 5 μg/ml), and A was kept as a control. Entry of *trp* was measured from A. At 35 min both A and B were gently diluted 500 times into fresh broth: A into D (the control) and B into C to reduce the NAL concentration to 0.01 μg/ml. Flasks C and D were then assayed for *trp* and *his* entry. (Portions from flask B were blended for *trp* entry to prove that 5 μg/ml completely inhibited transfer for 110 min.)

The results (Fig. 8) show that in flask D, the control, both *trp* and *his* enter with their normal entry times, 35 and 54 min, respectively. Were the 10-min NAL pulse inhibition reversible, in flask C *trp* would be expected to enter at 35 + 10 + 8 = 53 min and *his* at 54 + 10 + 8 = 72 min. In fact, *trp* enters at 73 min and *his* at 92 min. Furthermore, note that the delayed *trp* and *his* entry times in flask C are just their normal entry times plus 35 min; in addition, both markers rise rapidly to the level of their controls. In other words, the entry curves after dilution of NAL are isomorphic to entry curves obtained in normal pulse matings. Similar results are obtained for NAL pulses applied at different times during the mating. These results suggest that after a pulse of NAL, chromosome transfer is irreversible in the normal sense but that a new round of transfer, beginning at the sex factor, is initiated in the existing mating pairs.

One criticism of the above interpretation is that perhaps the retransfer in Fig. 8 occurs not in those cells which were in the process of transfer when NAL was added, but in a different fraction of the mating population. For example, one could imagine that in a population of paired bacteria only a fraction of the cells normally initiate chromosome transfer. The NAL pulse might irreversibly stop transfer in this fraction while inducing other cells to transfer normally.

To test this possibility, a mating was performed between Hfr Cavalli and MH1001 (AB1133 *Nal*') under conditions which allow measurement of the total entry curves and plateaus (Fig. 9). (The females are prestarved, and matings are carried out in M-9 minimal medium plus glucose with very little broth present.) After an early marker (proline) had completely entered, the culture was exposed to a 10-min pulse of NAL and diluted; portions of the diluted mating mixture were blended for assay of the early marker and for a distal marker (arginine). Arginine entry occurs normally at 30 min (Fig. 9) in this system, whereas it is delayed until 60 to 65 min in the NAL-treated culture; the fraction of the arginine marker retransferred is 53% in this experiment. If this retransfer were in a second population of cells, we would expect to see a 53% rise in proline recombinants (at about 48 min) in the NAL-treated culture; however, no increase was observed. This result makes it likely that the retransfer occurred in the same fraction of cells which had previously transferred proline.

The result that the plateau level of proline recombinants in the NAL-treated culture does not increase appreciably, even though 53% of the cells contain a second copy of the proline gene, argues that recombination must be efficient under these conditions.

**DISCUSSION**

The male and female roles in determining inhibition of chromosome transfer by NAL have been separated by complementary matings of *Nal* and *Nal* strains. Interrupted matings in
which recombinants are the end result, zygotic induction experiments, and transfer gradient measurements all demonstrate that NAL acts chiefly on the male bacterium when it stops chromosome transfer. This inhibition is observed both for early markers (transferred at 8 min) and for late markers (transferred at 55 min). These results are in agreement with Barbour (3) and Hol-
lom and Pritchard (23) and with the recent reports by Fisher and Fisher (17) and Bresler, Lan-
lzov, and Lukjaniec-Blinkova (8). The latter group has published curves (for one marker only) comparable to Fig. 3.

When DNA synthesis in the female cell is inhibited by 85%, there is little effect on marker entry or on the gradient constant, k. This result suggests either that little DNA synthesis is required in the female bacterium during the steps of union and chromosome transfer, although it may take place later, or that this type of DNA synthesis is not inhibited by NAL.

The mechanism by which NAL inhibits transfer in the male cannot be specified yet as the mechanism by which it inhibits DNA synthesis remains unknown. NAL primarily inhibits DNA synthesis (i.e. in the absence of protein synthesis) and DNA degradation occurs under conditions leading to lethality; protein synthesis is necessary for both degradation and lethality. Recently Boyle, Cook, and Goss (7) showed that NAL does not inhibit the action of crude cell-free extracts of DNA polymerase, deoxyribonucleotide kinase, or deoxyribosyl transferase; not does it cross-link DNA in bacterial strains which behave the same as Hfr H and PA309 under NAL treatment.

Several possibilities for the action of NAL in halting chromosomal transfer can, however, be ruled out. One of these is that NAL causes rapid separation of preformed bacterial pairs. Pairs remain stably joined in the presence of NAL, at least for periods long compared to the rapid interruption of transfer.

One could also argue that NAL inhibits transfer by degradation of the male chromosome— that either nicking or more extensive single-strand degradation of the male chromosome prevents transfer. Chromosome transfer is stopped within 1 or 2 min by 5 μg of NAL per ml. Since the rate of chromosome transfer is about 5 × 10⁴ nucleotides/min, one would expect nicking to occur, on the average, at least every 10⁴ nucleotides. The resolution of the alkaline-denatured sucrose gradient experiment (Fig. 1) is sufficient to observe this degree of nicking; since no such effect was observed, these results suggest that random nicking of the Hfr chromosome is not responsible for the inhibition of transfer by NAL.

It is quite possible that nicking occurs at a special place, such as the replication fork; such nicking would not be observed by this assay. Or, since the chromosome is probably transferred as a single DNA strand (11, 28, 33), this strand might be highly sensitive to breakage after NAL treatment. If this latter is the case, the transferred strand must be rapidly made insensitive to NAL treatment in the female, perhaps by some form of NAL-independent replication to the double-strand state.

The normal gradient constant k can be identified with random lesions in the donor chromosome which result operationally in chromosome interruption during transfer (37); under normal conditions there are about seven such breaks per chromosome. In NAl donors, there is a threefold increase in k at a NAL concentration of 0.5 μg/ml; this increase in k would correspond to the induction of about 15 extra transfer breakage lesions. The fact that removal of NAL does not allow simple resumption of transfer but requires a reinitiation of transfer suggests that NAL-induced lesions are not immediately repairable. However, since NAL causes little lethality at these concentrations, such lesions are ultimately lost or repaired.

The possibility that NAL causes nonfunctional genes to be synthesized which can be transferred but not expressed in the recipient is unlikely. If NAL caused gene inactivation either at the replicative fork or uniformly over the chromosome, male lethality should be high at NAL concentrations which cause rapid inhibition of transfer (e.g., 5 μg/ml); since male lethality is not observed, either NAL does not cause synthesis of nonfunctional genes or these are rapidly repaired.

The latter is true, repair would also be expected in the resistant female after transfer of nonfunctional markers.

The conclusion that NAL removal does not lead to resumption of transfer but to initiation of a new round of chromosome transfer is different from the conclusion of Holom and Pritchard (23). They studied transfer of only one marker with infrequent (5 min) blending, and it was not possible to differentiate between a lag induced by NAL and a new round of transfer.

Inhibition of DNA synthesis in bacteria by either NAL or thymine starvation produces many similar physiological effects (6; Winshell, Ph.D. Dissertation). In exponentially growing cells, both interruption of DNA synthesis by NAL (30, 35) or by thymine starvation (31) can result in induction of new growing forks beginning from the normal origin of bacterial replication.

Reversibility experiments, utilizing several markers, such as those described here with NAL have not been done with thymine starvation. In mating experiments between thymine-starved male and female cells, Pritchard (29) and Ishibashi (24) observed an inhibition (approximately 50%) of recombinant production (transfer). Upon supplementing the culture with thymine, recombinant production increased after a lag of about 5
min. This result was interpreted by Pritchard as a reversible inhibition of transfer. Since the marker entry time for Pritchard’s system is 8 min and his blending period was 5 min, conclusive evidence on the nature of reversibility after thymine starvation must await further experiments.

Since the completion of this work, a paper by Bouck and Adelberg (4a) has appeared with very similar results and conclusions. The present paper has extended their work to include studies on pair formation, alkaline sucrose gradient analysis of DNA after NAL treatment, and further analysis of retransfer of the chromosome after interruption of DNA synthesis with NAL.

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