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Events Occurring Near the Time of Synapsis During Transformation in *Diplococcus pneumoniae*

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A marker-specific and strongly temperature-dependent reaction was observed to occur at a time during transformation in *Diplococcus pneumoniae* after the donor deoxyribonucleic acid (DNA) had acquired single-strand properties and immediately preceding the integration of these strands into the recipient chromosome. Operationally, it was observed as the prevention of an intracellular inactivation process, also described in this paper, which is specific for low molecular weight or for damaged DNA, and which occurs if the recipient cells are held at suboptimal temperatures after the DNA has entered. Brief exposure of the cells to a higher temperature stabilized the DNA against this inactivation, in a two step process. It is the first step which has a strong temperature dependence ($\Delta H\ddagger$ = 70 kcal/mole, $\Delta S\ddagger$ = 160 entropy units), is marker specific, and which appears to be reversible. The second step is much less temperature-dependent and overlaps in time the start of integration. The enthalpy and entropy of activation are both consistent with those needed to open a loop of six to eight base pairs in a DNA duplex. It is suggested that these observations may reflect, and provide an assay for, the kinetics of synapsis, which on this model is limited in rate by the appearance of unpaired regions on the recipient duplex.

We report two phenomena which provide tools for study of intermediate reactions during transformation in *Diplococcus pneumoniae*. They occur after the donor deoxyribonucleic acid (DNA) has reached the eclipse state, in which its characteristics in this sytem are largely those of single strands (6, 10, 11), and before it has replaced a recipient strand to form the donor-recipient hybrid in the integration process (5, 9). The first phenomenon is an intracellular inactivation process, scored as a loss of potential transformants when the recipient cells are held at suboptimal temperatures after DNA entry. This inactivation occurs only when the donor-DNA fragments are short or when longer DNA fragments have been damaged, as by ultraviolet (UV) irradiation.

The second phenomenon to which we wish to direct attention is that the above inactivation may be prevented by a brief exposure of the cells to a higher temperature. This reaction, which we call stabilization, occurs during the time period when the donor strand must locate its homologous region on the recipient chromosome, and its properties, to be presented, suggest that it may coincide with the initial pairing event prerequisite to subsequent stages of recombination.

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MATERIALS AND METHODS

Media, bacterial strains, markers, and the preparation, shearing, and characterization of DNA were as described elsewhere (4).

Most DNA preparations carried two markers, resistance to streptomycin (*str-r1*) and novobiocin (*nov-r*), or erythromycin resistance (*ery-r2*) and *nov-r*. Except where noted, results were the same within experimental precision for all markers, though data are often shown only for one. Many experiments used large quantities of DNA, requiring the preparation and shearing of several batches. All preparations showed single-strand molecular weights approximately one-half those of the double-strand fragments, as determined from median sedimentation coefficients ($S_{20,w}$) measured in an analytical ultracentrifuge (15).

Transformation procedures are mostly described in the figure legends of particular experiments.

Competent cells were prepared as described by Porter and Guild (12). To avoid uncertainty as to time for expression or multiplication of transformed cells, in all experiments except those of Table 1 appropriate dilutions were plated in nonselective agar, allowed to express for 2 hr at 37 C, and then overlaid with drug-containing agar.

Procedures for recovery of donor activity in lysates of recipient cells were modified from those of Ghei and Lacks (6). The initial exposure of the cells to DNA was always at 25 C for 5 min. Frozen competent cells were used in some experiments, but in all of those shown here, except in Fig. 3b, cells were freshly grown to competence in 150-ml batches. When the culture had grown to about 2×10^7 colony-forming units/ml, it was transferred to ice for 1.5 min and then to 25 C for 2.5 min, at which time donor DNA was added to a final concentration of 3 μg/ml. After 5 min at 25 C, pancreatic deoxyribonuclease was added to a final concentration of 10 μg/ml in 0.01 M $MgSO_4$. Portions were diluted sixfold into growth medium at 0, 25, 30, 37, or 40 C, and 30-ml samples were transferred to ice at intervals. Three persons together could perform recovery curves at three temperatures simultaneously from one batch of cells, allowing precise normalization to a single control value, usually taken as the activity found after 40 min at 25 or 30 C.

Extracts of these samples were prepared by centrifuging the cells, washing them twice in SSC (0.15 M NaCl, 0.015 M sodium citrate), and resuspending the cells in 0.2 ml of a solution containing 0.15 M sodium citrate, 0.15 M NaCl, and 0.1% sodium deoxycholate. After 5 min at 37 C, the cells had lysed, and they were then diluted 10-fold with 0.15 M NaCl and frozen. Each 1 ml of lysate at this stage represented 2.5 ml of the original culture containing approximately 0.8 μg of recipient-cell DNA. Donor activity was assayed by adding 2.0 ml of fresh recipient cells to 0.1 ml of lysate for 30 min at 33 C before addition of deoxyribonuclease, dilution, and plating as above. At this stage, the total DNA concentration was about 0.04 μg/ml.

UV irradiation at 254 nm was delivered from a low-pressure germicidal lamp to DNA at a concentration of 10 to 20 μg/ml in saline-citrate, with stirring, to the survival levels indicated in the Results section.

RESULTS

In preliminary experiments, it was found that the relation between transforming activity and length of donor DNA is sensitive to the temperature at which the cells are exposed to the DNA, but not strongly so (Table 1). These tests had been designed to see whether the magnitude of the excluded length, a part of each donor molecule unavailable for recombination (4, 8), could be altered by slowing down integration. If the excluded length represented a region degraded from the end of each donor fragment by an exonuclease, and if the nuclease were acting continuously, one might expect a drastic change in the activity of a DNA fragment near the minimum length as the conditions changed.

The results in Table 1 show changes in the direction to be expected if such a nuclease had greater opportunity to act at lower temperatures, even if at a slower rate. However, because the activity falls very rapidly for small decreases of DNA length in the region near 500 nucleotide pairs (4), they could be accommodated in the analysis by a change of only 15 to 20 nucleotides out of 400 to 450 in the apparent excluded length. Over the range from 25 to 37 C, therefore, the excluded length appears to vary only within narrow limits.

There is, however, a readily observed effect of temperature on the activity of short DNA fragments. To examine this point further, we held the temperature constant during entry of the DNA, eliminating one variable in the data of Table 1, and then varied temperature after termination of entry, observing the effects on: (i) the ultimate appearance of transformants in the culture initially exposed to the DNA and (ii) the recovery of donor transforming activity

TABLE 1. *Transforming activity as a function of length of DNA, assayed at various temperatures*[a]

Size of DNA fragments		Relative activity[c] at T =		
Mol wt	L[b]	37 C[d]	30 C	25 C
0.33×10^6[d]	500	0.0016	0.0010	0.0004
0.6×10^6	900	0.028	0.020	0.011
1.5×10^6	2,300	0.25	0.25	0.22
1.9×10^6	2,900	0.31	0.30	0.26
12×10^6	18,000	1.00	1.00	1.00
		1.2[e]	1.0[e]	0.5[e]

[a] Previously frozen competent cells were exposed to 0.01 μg of DNA/ml for 15 min at the temperature indicated. Deoxyribonuclease was added, and the cells were transferred to 37 C for 75 min to allow phenotypic expression before plating to score for transformants to *nov-r* (data shown) and *ery-r* (similar results). Under these conditions of limiting DNA, the weight uptake of DNA by the cells is expected to be nearly independent of length at 30 C (3).

[b] Nucleotide pairs.

[c] Activity relative to that of 12 million molecular weight sample = 1.00, which represents about 3×10^5 transformants/ml at 30 C in the conditions used here. The lowest activity listed represents 60 transformants/ml.

[d] This sample is 7.2*S* DNA isolated from the peak of the distribution in a preparative velocity sedimentation run by the method of Cato and Guild (3).

[e] Activity of 12 million molecular weight donor ($S_{20,w}$ = 26) at temperature T relative to its activity at 30 C.

in lysates of such cultures. The findings were as follows.

Decline. The number of transformants produced by short DNA fragments, but not by long ones, declined slowly when the cells were held at 25 C after exposure to DNA, before being eventually transferred to 37 C for phenotypic expression and scoring. The extent of this intracellular inactivation, to which we shall refer as *decline*, increased as the length of the DNA decreased (Fig. 1a). The result was the same whether exposure was terminated by the addition of deoxyribonuclease or of excess competitor DNA, showing that the inactivation was not due to penetration by extracellular deoxyribonuclease (data not shown). Decline occurred to a lesser extent but was significant when cells were exposed to DNA at 30 C and held at 30 C (Fig. 1b), and the decline at 25 C was less when the initial exposure to DNA was at 30 C (compare Fig. 1a and 1b). If the cells were chilled rapidly by dilution into cold medium and rewarmed rapidly to 37 C when transferred back, there was no decline at 0 C for at least 1 hr (N. Shoemaker, *unpublished data*), whereas a small decline was seen when cooling and rewarming were less rapid (Fig. 1c).

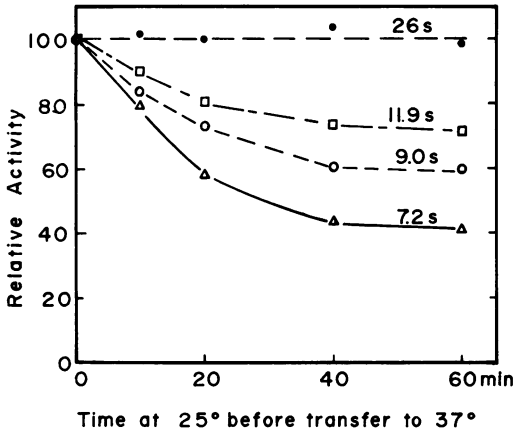

FIG. 1a. *Loss of potential transformants (decline) when cells were held at 25 C after exposure to DNA of various lengths. Competent cells were exposed for 5 min at 25 C to 1 μg/ml of DNA preparations of the indicated $S_{20,w}$, deoxyribonuclease was added, and samples were transferred to 37 C at the times shown. After 20 min at 37 C, the samples were diluted, plated, and later overlaid with selective drug as described in Materials and Methods. Nov-r data are shown. Symbols: ●, 26S; □, 11.9S; ○, 9.0S; △, 7.2S. Activity at zero time depends strongly on DNA length (compare Table 1). All points represent at least 100 colonies counted. Previously frozen cells were used here; similar results are found with fresh cells.*

At 25 C, the decline reaction was half complete in 12 to 15 min, and ceased by 30 to 40 min, leaving a substantial residual activity of about 40% for 7.2S DNA. Prefractionation of such DNA by velocity sedimentation in a sucrose gradient, to remove longer fragments,

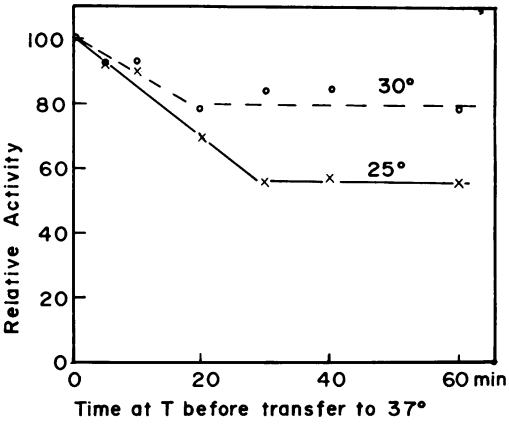

FIG. 1b. *Decline at 25 C and at 30 C when initial exposure of cells was at 30 C for 10 min, to 1 μg of 7.2S DNA per ml. Procedure was similar to that of Fig. 1a, except that, after addition of deoxyribonuclease, half of the sample was transferred to a 25 C bath and half left at 30 C for times indicated. Nov-r data are shown; ery-r results were similar. Symbols: ○, 30 C; ×, 25 C.*

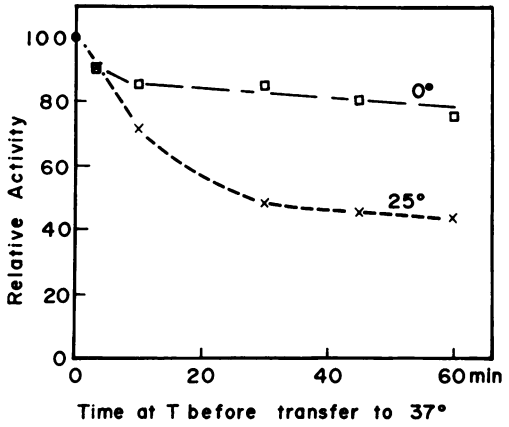

FIG. 1c. *Effect of holding cells at 0 or 25 C on the loss of potential transformants from short DNA fragments. Cells were exposed for 5 min at 25 C to 1 μg of 7.7S DNA/ml, before addition of deoxyribonuclease. Part of the culture was then transferred to an ice bath. At times indicated, samples were transferred to a 37 C bath for 20 min and plated as in Materials and Methods. Str-r data are shown. Symbols: □, 0 C; ×, 25 C. As noted in text, more recent data of N. Shoemaker suggest that the small amount of inactivation seen here at 0 C occurred during the cooling or rewarming steps.*

did not change the final activity remaining after decline.

Cells transformed by intact high-molecular-weight DNA showed no loss of potential transformants on holding them as long as 3 hr at 25 C, but when the DNA had been irradiated by UV light there was decline (Fig. 2a). At 10% survival, there was a slow loss of about 15% of the remaining activity, and at 0.25% survival, 70% of the activity disappeared on holding the cells at 25 C after entry. UV irradiation also increased the extent of decline for 9*S* DNA (Fig. 2b), and in both Fig. 2a and 2b the final level is lower than that seen for 7.2*S* unirradiated DNA.

We have so far seen little difference among the markers tested in their susceptibility to decline.

Integration. Because the above results suggested that the inactivation and integration reactions may compete for donor DNA, we measured in our system the kinetics of integration, as defined operationally by the recovery of donor transforming activity from eclipse in lysates of recipient cells. Ghei and Lacks (6) and Lacks et al. (11) showed that by several criteria the increase in activity paralleled the conversion of donor DNA from single-strand to double-strand properties, and it is this conversion that we call *integration* here.

For freshly grown competent cultures and 26*S* donor DNA, the half times for recovery from eclipse were 12 to 13 min at 25 C, 7 min at 30 C, and 3 min at both 37 and 40 C (Fig. 3a). These times were similar for *str-r*, *ery-r*, and *nov-r*, all high-efficiency markers in the recipient strain, Rx-1. Cultures frozen at high competence and thawed for use in such experiments showed a lag of 1.5 to 2 min, followed by kinetics very similar to those of fresh cultures, as illustrated in Fig. 3b for two temperatures. Data are shown in Fig. 3b for 26*S* and for 11*S* DNA, the smallest size with sufficient activity to be assayable in lysate experiments. The conclusion is that the kinetics of integration are not detectably dependent on the length of the DNA. UV irradiation of the donor DNA, however, caused a 2- to 3-min delay of integration at 25 or 30 C, as shown in Fig. 3c. A similar delay has been seen at 37 C in other experiments.

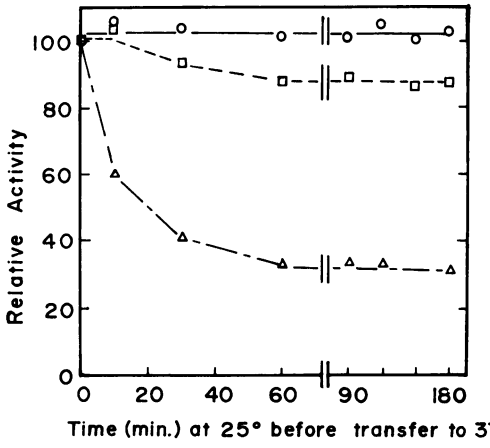

FIG. 2a. *Intracellular loss of transformants from UV-irradiated high-molecular-weight DNA. 26S donor DNA was irradiated at 254 nm as described in Materials and Methods, to survival levels of 10 and 0.25% (relative activities at zero time in this plot), and tested for intracellular decline at 25 C as in Fig. 1a. Str-r data are shown. Symbols: O, unirradiated control; □, 10% survival; △, 0.25% survival.*

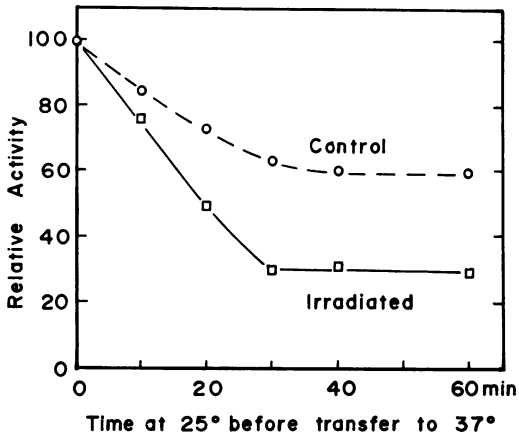

FIG. 2b. *Effect of UV irradiation of 9S DNA on intracellular decline at 25 C. Cells were exposed for 10 min at 25 C to control or to UV-irradiated 9.0S donor DNA, and tested for intracellular decline on holding at 25 C as in Fig. 1a. The irradiated DNA has 8% of the control activity at zero time on this plot. Nov-r data are shown. Symbols: O, unirradiated; □, irradiated.*

Integration occurred at 25 C to the same final level as at 30 C for high-molecular-weight DNA (Fig. 3a), but to a somewhat lower level for 11*S* or for UV-irradiated 26*S* DNA (Fig. 3b and 3c). The lower level for these DNA preparations reflects some decline at 25 C (compare Fig. 2a). At 37 C and at 40 C, the donor activity continued to rise, paralleling DNA replication which occurred under these conditions in our system (*unpublished data*).

Stabilization. With frozen cultures and at 25 C in fresh cultures, there was a distinct lag before recovery from eclipse began. The half times for integration and the decline reaction were similar at 25 C, consistent with the hypothesis that integration protects against de-

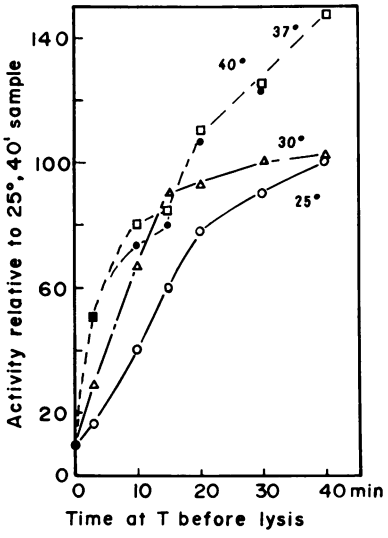

FIG. 3a. *Recovery from eclipse of donor marker activity in lysates of freshly grown recipient cells. Cells were exposed for 5 min at 25 C to excess 26S str-r, nov-r DNA, treated with deoxyribonuclease, and at 0 min transferred to the temperatures indicated, as described in detail in Materials and Methods. Data here represent str-r transforming activities in lysates prepared at times indicated, and are the averages of five assays on two batches of lysates, all of which gave similar curves; "100" represented 1,102, 1,791, 554, 1,587, and 773 colonies in the respective assays for str-r. Similar results were seen for nov-r, in fewer assays, and for ery-r (one experiment). Symbols: O, 25 C; Δ, 30 C; □, 37 C; ●, 40 C.*

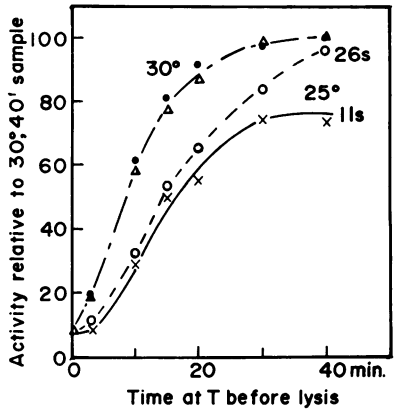

FIG. 3b. *Recovery in lysates of str-r activity in 11S donor DNA compared with that in 26S DNA. In these experiments, previously frozen competent cells, in 10% glycerol, were used instead of freshly grown cells as in Fig. 3a. There is a lag of 1.5 to 2 min characteristic of frozen cells; otherwise the curves are essentially superimposable on those of Fig. 3a. Cells were thawed for 10 min at 37 C, iced for 1 min, transferred to 25 C for 3 min, and then exposed to DNA at 25 C for 5 min. Subsequent procedures were the same as for Fig. 3a. Symbols: ×, 11S, 25 C; O, 26S, 25 C; Δ, 26S, 30 C; ●, 11S, 30 C.*

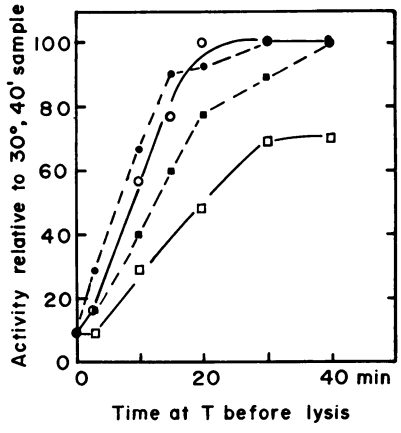

FIG. 3c. *Delay in recovery from eclipse induced by UV irradiation of donor DNA. The 26S DNA used in Fig. 3a was irradiated to 8.5% survival and tested for the kinetics of recovery from eclipse at 25 C and at 30 C in freshly grown cells as in Fig. 3a. Data shown are for str-r and are the averages of three assays which agreed within 0.5 min for the half times. Open symbols, irradiated; closed symbols, control data from Fig. 3a; circles, 30 C; squares, 25 C.*

cline. To see if there was a detectable reaction prior to integration, however, we asked whether the potential transformants could be stabilized against decline while still in the lag phase of the integration process. Cells from a frozen competent culture were exposed to low-molecular-weight (8.9*S*) DNA at 25 C, treated with deoxyribonuclease, and then pulsed briefly to higher temperature before being put back at 25 C for 1 hr to undergo decline. The results (Fig. 4a) show a rapid stabilization occurring in seconds at 37 C, followed by a distinct second step completed in 1 to 2 min. After this time, the same number of transformants was observed whether or not the cells were subjected to holding for 1 hr at 25 C. The level of the first step was consistently different between markers, and the timing of the second step appeared to differ also, *str-r* always rising furthest and fastest, *ery-r* least and slowest, with *nov-r* intermediate. As described in the legend to Fig. 4a, the marker differences and the intermediate plateau were statistically reliable and were repeatedly seen, in the three experiments whose averages are presented here and in others both before and after these. The level of the first step did vary, however, in these three and in other experiments, such as that in Fig. 4b where the effect of UV irradiation on

stabilization of 9S DNA is shown. The irradiated DNA showed the two-step stabilization phenomenon, but, compared with the control assayed at the same time, the level of the first step was lower and the second step appeared to be spread over a longer period of time.

Although we cannot define the half time of the first step with precision, it appears to be not more than 10 sec and perhaps as short as 5 sec at 37 C. The upper limit would be 15 to 20 sec *if* the rate were uniform while the temperature rises, which it is not. At 30 C, this step was much slower, but still showed a difference between markers (Fig. 4c). The half time would appear to be about 90 sec if one divides the reaction into two steps, as we believe is correct, or 2 min if the entire stabilization is regarded as one reaction. These times are 10- to 20-fold longer than those at 37 C. If the second part of the reaction is comparable to that at 37 C, its time course is not greatly different at the two temperatures, except that it is delayed by the slowness of the first step.

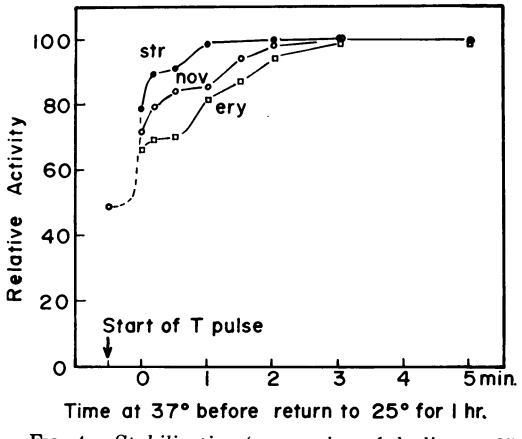

FIG. 4a. *Stabilization (prevention of decline at 25 C) by brief exposure of cells to 37 C. Competent cells, previously frozen and thawed, were added to a set of tubes containing 8.9S DNA, carrying all three drug-resistance markers, at 25 C. Deoxyribonuclease was added after 5 min. Controls were left at 25 C for 1 hr. The rest of the tubes were transferred to a 40 C bath with shaking (in a rack) for 30 sec, at which time prior tests had shown that they had just reached 37 C, and then were transferred rapidly to a 37 C bath. At the times indicated tubes were plunged into ice water for 10 sec and transferred to 25 C for 1 hr to undergo decline, before returning to 37 C and plating as in prior experiments. The 100% value represents a control held at 37 C for 10 min after pulsing, and then plated without returning to 25 C. In separate tests of events during the 40 C pulse, it was found that little stabilization occurred until shortly before the cells reached 37 C (compare Fig. 4c at 30 C), approximately as indicated by the dotted line. Symbols: ●, str-r; ○, nov-r; □, ery-r. Data shown here are the averages of three separate experiments, each of which showed the step at 10 to 30 sec and the differential between the markers at these intermediate time points, but not at either the starting (48%) or final (100%) control levels. Markers at a given time point are scored from multiple platings of cells in a single tube, and systematic differences are reliable. The 100% value represents 1,200 to 1,300 colonies counted in the three experiments together.*

DISCUSSION

Two previously unknown phenomena have

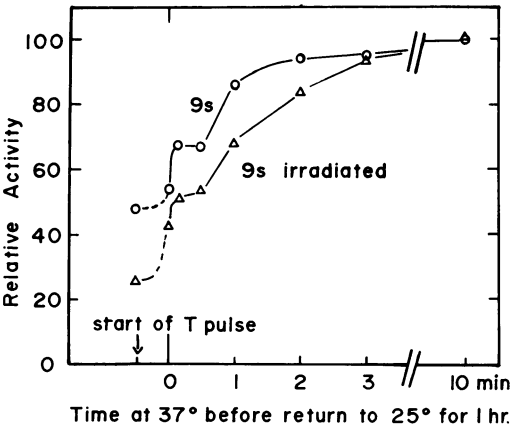

FIG. 4b. *Stabilization of UV-irradiated 9S DNA compared with unirradiated control. Procedures were identical to those in Fig. 4a except that the DNA preparations used were 9.0S, UV-irradiated to 20% survival or unirradiated. Data are for nov-r. Symbols: ○, unirradiated; △, irradiated.*

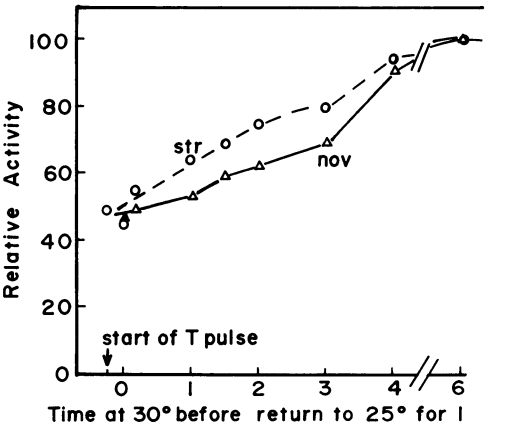

FIG. 4c. *Stabilization at 30 C against decline at 25 C. Similar to Fig. 4a except that DNA was 9.1S, str-r, nov-r, and cells were brought from 25 C to 30 C by shaking in 37 C bath for 14 sec before transfer to 30 C bath. For quick return to 25 C, tubes were plunged in ice for 5 sec before transfer to 25 C. Symbols: ○, str-r; △, nov-r.*

been found in these experiments and provide tools for further study of intermediate steps in transformation. The reaction we call decline is a loss of potential transformants, occurring after the DNA is insensitive to external deoxyribonuclease, when the cells are held at temperatures below 37 C. Since we usually expose cells to DNA at 25 or 30 C for several minutes, and frequently longer, it may be presumed that some inactivation of lower molecular weight or damaged DNA occurs during the exposure time, prior to the 0-min point of the experiments in Fig. 2. By definition, we cannot observe decline at 37 C, since that is the control condition, but there may be such effects at this temperature also. A practical point worth noting for procedures, therefore, is that transforming activity from short or damaged DNA fragments may be lost by inadvertent cooling of the cells during manipulation.

For the present discussion, the mechanism of inactivation which produces decline is not of critical importance, and we defer its consideration. We use decline merely as a tool for detecting stabilization, which represents a change in the state of the DNA such that it is no longer subject to inactivation.

Stabilization against decline precedes integration at both 30 and 37 C, as may be seen by comparing Fig. 3 and 4. At 37 C, the first step is complete almost as soon as the cells reach 37 C, whereas at 30 C it takes about 3 min for completion. By this time, at 30 C integration is less than 30% complete in freshly grown cells, and less than 20% complete in previously frozen cells of the type used for the stabilization experiments (see Fig. 3b and 4c). The second step of stabilization overlaps in time the beginning of the recovery from eclipse.

At 25 C, our only measure of stabilization is in terms of cessation of decline. At 37 and 40 C, however, a later step of integration is rate-limiting, since recovery from eclipse shows very similar half times at both temperatures (Fig. 3a). At 30 C, integration takes about 4 min longer than at 37 or 40 C, comparable to the increased time for completion of stabilization (Fig. 4c). The suggestion is that the subsequent steps of integration are not strongly temperature-dependent and that at 25 C the half time for recovery from eclipse may be determined primarily by the half time for the first step of stabilization. To test this implication, the half times were converted to apparent first-order rate constants, expressing the probability per unit time that in a given cell the reaction occurs, and compared against reciprocal absolute temperature on an Arrhenius plot (Fig. 5). The good fit of the data to a straight line implies that the rates of stabilization at 37 and 30 C and of integration at 25 C do indeed reflect a common rate-limiting reaction with the rather high activation energy, calculated from the slope, of 70 kcal/mole (Q_{10} $\sim$ 50). Absolute rate theory gives $\Delta H\ddagger$ = 70 kcal/mole, $\Delta S\ddagger$ = 160 entropy units, with an estimated range for each of not more than $\pm$ 20%.

We can now give a more explicit interpretation of the final level of activity after decline; namely, it represents that fraction of the DNA stabilized irreversibly, by initiation of integration, before there occurs an inactivating event which is more probable the shorter the DNA. At 30 C, more DNA escapes inactivation than at 25 C, but at both temperatures integration occurs and inactivation ceases (compare Fig. 1b and 3b). At 0 C, little or nothing of significance happens to the DNA, and on return to 37 C integration proceeds normally.

From the shape of the stabilization curve at 37 C, it is clear that the process in the culture as a whole occurs in at least two steps. The intermediate plateau, differing in level for the various markers, implies either that all of the donor DNA has reached a new state in which it is partially stabilized, or that subsets of the donor molecules, perhaps in different groups of cells, follow different kinetics towards stabilization. These kinetics, in turn, could reflect different starting states on a common pathway or separate noninterchangeable paths. To distinguish these alternatives will require further experiments of a different kind.

The observed decline of high-molecular-weight UV-irradiated DNA and the increased decline seen with 9*S* irradiated DNA lead to the conclusion that at 37 C cells successfully integrate damaged DNA which would have been inactivated on holding at 25 C. Both the lower level of the first step of stabilization (Fig. 4b) and the brief delay of integration of irradiated DNA suggest that pyrimidine dimers inhibit completion of stabilization and permit a greater loss of markers than would have occurred under optimal conditions. This result for *D. pneumoniae* is consistent with the report that dimers are integrated and later excised during transformation of *Haemophilus influenzae* (14).

The observation that UV-irradiated high-molecular-weight DNA shows decline like a low-molecular-weight DNA could be explained if the dimer-specific endonuclease acts on at least some of the dimers at a stage prior to integration. However, this enzyme, as found in

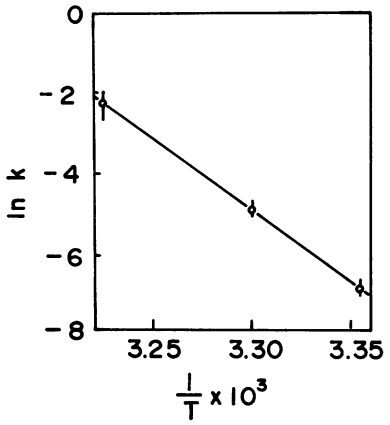

FIG. 5. *Arrhenius plot (logarithm of rate constant against reciprocal absolute temperature) comparing rates of first step of stabilization at 37 C and at 30 C to rate of integration at 25 C. Because the data represent probability distributions for a single event occurring per cell, the results may be compared as apparent first-order rate constants, k = (ln 2)/(half time). Circles represent half times of 7, 100, and 700 sec at 37, 30, and 25 C, respectively; and the ranges indicated are 5 to 10 sec, 90 to 120 sec, and 10 to 15 min.*

Micrococcus luteus, acts preferentially on double-stranded DNA (3), and the further interpretation of this observation must await more evidence on the mechanism of the inactivation process causing decline.

Major experimental observations. The major experimental observations may be summarized as follows. There is an intracellular inactivation process detectable only with short or with damaged donor DNA fragments. This process is terminated by a biphasic reaction, the first step of which precedes integration, is marker-specific, and is strongly temperature-dependent, with $\Delta H‡ = 70$ kcal/mole, $\Delta S‡ = 160$ entropy units. The second step is less temperature-dependent, overlaps in time the early part of the integration curve, and renders stabilization complete.

Interpretation of stabilization as synapsis. The findings on decline and stabilization as discussed above are rather straightforward. Although an interpretation of the stabilization reaction in molecular terms must be more speculative at this stage, the results suggest a model of sufficient interest that it should be pointed out as a stimulus for further investigation.

We note that: (i) high activation energies suggest cooperative reactions, such as the denaturation of macromolecules; (ii) marker specificity strongly implies a reaction involving the chromosome, rather than, for example, a solubilization of the membrane; and (iii) stabilization occurs after entry and prior to integration, during the time when donor strands have to locate homologous regions on the chromosome.

We suggest, therefore, that the reaction we observe as the first step of stabilization may be in fact the initial synapsis of donor and recipient DNA, limited in rate by the local denaturation of the recipient helix over a region long enough to expose the bases and allow the donor strand to form a tentative reversible association with its complement (Fig. 6). The second step of stabilization could then coincide with the initiation of further displacement of the recipient strand, probably with the aid of one or more enzymes, preventing further reversal of the association. If there were no preexisting break at the site of pairing, at least one endonucleolytic cleavage would be needed. Exonucleolytic removal of the recipient strand would provide a simple mechanism for further integration.

On this model, the activated state is a locally denatured region of DNA, and with some reservations one may equate the activation energy to the heat of formation of coil from helix; 70 kcal/mole corresponds to the enthalpy of melting 8 ± 1 average base pairs from an end, or perhaps 7 ± 1 in an interior loop where one extra stacking interaction must be broken (2, 13). The entropy per base pair may be estimated from $\Delta S° = \Delta H°/T_m$ as 25 entropy units, whence $\Delta S‡ = 160$ entropy units corresponds to the entropy of opening six or seven base pairs. The agreement is close enough at this stage.

These estimates of the length of the region of association imply that the complex should be unstable (17), though with a finite lifetime during which it could be recognized and acted upon by enzymes (7). This implication is con-

FIG. 6. *Pairing in transformation as suggested by the kinetics of the stabilization reaction. Single-stranded donor DNA, carrying a marker sequence indicated by the dot, pairs reversibly with a short region of activated chromosome. Extension of the region of pairing renders stabilization complete and initiates integration. The activated region may be a simple internal loop, as shown, or a loop bound to a third component X, or a short gap on one strand of recipient, provided only that the frequency of such regions is controlled primarily by the activation energy for opening a loop in double-helical DNA.*

sistent with the interpretation of the intermediate plateau of the stabilization curve as a partially stabilized state of all of the DNA, from which its probability of integration is greater at 37 C than at 25 C. The differences in stability of the markers could reflect at least two probabilities: (i) that in such a short sequence the pairing is uniquely positioned with respect to the rest of the DNA, and (ii) that, if so, the reaction goes forward rapidly, as influenced by the local sequence nearby.

Further detailed consideration of the idea of an activated recipient chromosome suggests that a system more complex than a simple "breathing" (16) may be required, including perhaps a DNA-binding protein similar to the T4 phage gene-32 product (1) or a component which excises short gaps with a finite lifetime before repair. If the rate of producing unpaired regions is controlled primarily by the activation energy for opening a segment of DNA helix, these systems would be compatible with our hypothesis that by observing stabilization we have a tool for measuring the rate of pairing in transformation and how it is influenced by a number of variables.

Relation between excluded length and decline. These investigations were started to test a simple hypothesis about the excluded length, namely, that an exonuclease could be acting continuously on one or both ends of a donor DNA fragment until integration rescued it. We found another reaction, decline, restricted to short DNA fragments or damaged ones, and that the excluded length itself is almost invariant with the temperature of assay (Table 1 and discussion in Results section). Inasmuch as the efficiency of completing integration is low for damaged or short DNA fragments, whether the reaction causing decline is related to that producing the excluded length cannot be decided yet. The latter retains its formal significance as a length of each donor strand unavailable for recombination, and may be due to a nuclease acting only briefly during entry, to the binding of a segment of DNA in an unavailable conformation, or to a reaction occurring late in the recombination process, such as, for example, an excision of part of the newly integrated donor before final ligase action.

Stabilization and the replication fork. A final point to be noted is that the stabilization reaction occurs rapidly, for all three markers studied, in a time of the order of 1% of a division cycle at 37 C. Even if these markers were close together on the chromosome, no degree of synchrony in cell cycle could produce such a result, and therefore stabilization must be occurring independently of the DNA replication fork in this system. On the other hand, if our interpretation of the reaction is correct, unpaired DNA at the fork could impart some bias toward integration at that point, particularly at lower temperatures. In a species or a mutant deficient in a component required to extend the lifetime of the activated state, such a bias could be more significant than it appears to be in our experiments.

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