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Early Stages in DNA Binding and Uptake During Genetic Transformation of Pneumococci

(divalent cation complexing agents/cell surface sites/nucleolytic splitting)

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ABSTRACT **Ethylenediaminetetraacetate and other divalent-cation-complexing agents greatly stimulate the cellular binding of DNA molecules to competent pneumococci, while the appearance of genetic transformants and nuclease-resistant DNA binding are completely inhibited. Based on this finding, we developed an experimental system in which three early and consecutive stages of genetic transformation can be experimentally separated: (*i*) attachment of DNA molecules to cell surface sites that are only demonstrable in the competent state; (*ii*) a divalent-cation-dependent nucleolytic splitting and release of the adsorbed molecules to the medium; and (*iii*) emergence of potential transformants accompanied by an energy-requiring and divalent-cation-dependent process in which the cell-associated DNA molecules become inaccessible to shearing forces, nucleases, anti-DNA serum, and polycations.**

Genetic transformation of pneumococci requires that the recipient cells be "competent" to bind and absorb extracellular DNA molecules. The molecular basis of this capacity seems to involve a subtle alteration of the cell surface, and by appropriate experiments it has been possible to identify several specific macromolecular factors that take part in this surface modification (1). While the sequential appearance of these factors seems clearly associated with the development of competence, it is not known at which stage and for what functions of the transformation process these factors are required.

Such an assignment of functions is hampered by the fact that the stages of the transformation process are not well separated in time: attachment of DNA molecules is rapidly followed by an unknown number of subsequent stages. In this communication we describe an experimental system that allows a clear, temporal separation of three consecutive early phases of genetic transformation. This was made possible by our finding that EDTA (and other divalent cation complexing agents) can selectively inhibit stages 2 and 3 of genetic transformation, while allowing DNA molecules to accumulate, attached to binding sites on the cell surface. Our report fully confirms and further develops the observations made by Morrison and Guild (2) and by Lacks (3) concerning the involvement of surface-located nucleases in transformation.

MATERIALS AND METHODS

In most of the experiments the laboratory wild-type strain of *Diplococcus pneumoniae* (R36A) was used. Most of the experimental methods have been described in detail in previous publications; these include growth of pneumococci in chemically defined medium (4), activation of cells to competence (5), determination of the viable titer and transformation frequencies of cultures, and preparation of transforming DNA (6). Antiserum against double-stranded DNA from the serum of a patient with lupus erythematosus was kindly provided by Dr. Robert Thoburn of this university.

Abbreviations: EDTA, ethylenediaminetetraacetate; EGTA, ethylene glycol bis(β-aminoethylether)-*N*,*N*′-tetraacetic acid; TCA, trichloroacetic acid; SA^r, sulfanylamide resistant; K^r, micrococcin resistant; $Strep^r$, streptomycin resistant.

RESULTS

DNA Binding in the Presence of EDTA. Our basic strategy has been to find a selective inhibitor or experimental condition that would allow normal DNA binding to the cells, but would prevent the nuclease-resistant association between molecules and cells and would also prevent genetic transformation. Published observations suggested that the appearance of nuclease-resistant form of DNA was an energy requiring process (1, 5, 7–10). Table 1 shows that, indeed, several inhibitors of cellular energy metabolism cause a greater decrease in the nuclease-resistant fraction of cell-associated DNA than in the total amount of DNA bound. However, of all the compounds tested, the most clear-cut selective inhibition was caused by EDTA, EGTA (ethylene glycol bis(β-aminoethylether)-*N*,*N*′-tetracetic acid), and other chelators of divalent cations. The appearance of nuclease-resistant DNA (and genetic transformation) was virtually completely inhibited by these agents, while, interestingly, total DNA binding was actually stimulated (5 to 10-fold) over the normal conditions. This double effect would be compatible with an inhibition by EDTA (and other chelating agents) of some intermediate steps in transformation leading to the accumulation of DNA molecules in the initial cell-attachment stage. For this reason, we proceeded to characterize this system in detail.

Characteristics of DNA Binding in the Presence of EDTA. The striking effect of EDTA on DNA binding is shown in Fig. 1. While in the absence of this agent total DNA binding, nuclease-resistant binding, generation of trichloroacetic acid (TCA)-soluble DNA fragments, and the accumulation of potential transformants all follow a closely parallel time course, in the presence of EDTA virtually all the cell-associated DNA accumulates in a nuclease-sensitive form, and all the other processes are inhibited.

Table 2 shows that, in the presence of EDTA, DNA molecules attach to the normal, competence-specific cellular sites: binding requires prior activation to the competent state, physiologically or genetically incompetent cells do not bind; single-stranded DNA binds only poorly, while double-

stranded homologous DNA can suppress binding in a competitive manner.

We examined the physical nature of DNA adsorbed to competent bacteria in the presence of EDTA: DNA-"loaded" bacteria were lysed by deoxycholate and the lysate analyzed by centrifugation in sucrose and cesium chloride gradients. It can be seen from Fig. 2 that the DNA reisolated was double stranded and had a molecular weight similar or identical to that of the donor DNA.

Completion of the Transformation Process upon Removal of EDTA. Competent pneumococci were loaded with [^{3}H]DNA (carrying the streptomycin resistance marker) in the presence of EDTA. Excess nonadsorbed DNA was removed (either by filtration or by several cycles of low speed centrifugation) and the bacterium-DNA complexes were gently resuspended in medium containing Ca and Mg and were incubated at 30°. Fig. 3 shows that readdition of the divalent cations resulted in essentially two phenomena: (*i*) a rapid loss of cell-bound DNA (to the medium) and (*ii*) an equally rapid emergence of potential genetic transformants in a nuclease-resistant form. The accumulation of transformants was accompanied by a parallel increase in the nuclease-resistant fraction of cell-bound DNA and by the production of TCA-soluble DNA fragments.

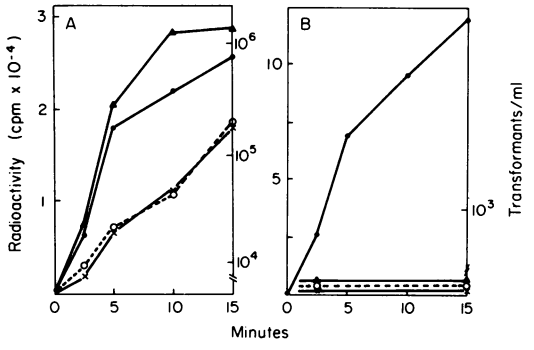


FIG. 1. *Kinetics of [^{3}H]DNA binding, production of TCA-soluble DNA fragments, and transformation in the presence and absence of EDTA.* Competent cells were suspended in SPA (0.15 M NaCl, 50 mM potassium phosphate buffer (pH 8), 1 mM $MgCl_2$, 0.3 mM $CaCl_2$, 2 mg/ml of glucose or sucrose, and 0.8 mg/ml of albumin) (7, 3) without EDTA (*A*) or with 10 mM EDTA (*B*). [^{3}H]DNA was added at 0 min, and incubation was at 30°. Samples were tested for total cell-bound DNA (●), DNA taken up in a nuclease resistant form (×), production of TCA-soluble DNA fragments into the medium (○), and *str*^{r} transformants (▲). Radioactive DNA was prepared by biosynthetic labeling: a thymidine-requiring and Strep^r strain of pneumococcus was grown in synthetic medium supplemented with [^{3}H]thymidine ([^{3}H]TdR) at 1.2 μg and 100 μCi/ml concentration. The specific radioactivities of DNA preparations were in the range of 2 to 6 × 10^6 dpm/μg of DNA. In order to minimize radiochemical damage, labeled DNA was used within 3–4 weeks after preparation. Rapid decline in transforming activity was noted in older (2 months) preparations. *Determination of cell-bound and released DNA and DNA fragments.* A suspension of competent cells (about 1.4 × 10^8 viable cells per ml) was incubated at 30° with radioactive transforming DNA (0.2 μg/ml). After exposure to DNA, the suspension was centrifuged in the cold (10,000 × *g*, 5 min). The bacteria were resuspended in SPA solution and were used to determine cell-bound DNA fractions and transformants; the supernatant was processed for the determination of low-molecular-weight DNA fragments. Portions of the supernatant (0.3 ml) were precipitated with 0.3 ml of cold 10% TCA, the precipitate was removed by centrifugation, and 0.1-ml aliquots of this supernatant were pipetted onto Whatman GF/A filter disks and used for determination of TCA-soluble DNA fragments. *Transformants and cell-bound DNA fractions.* A 0.1-ml aliquot of the cells resuspended in SPA was removed into 2.9 ml of growth medium containing 2–6 μg/ml of DNase I (Worthington) and scored for the number of transformants after 150 min incubation at 37°. The rest of the cell suspension was divided into two portions. One portion was centrifuged immediately, washed twice with SPA solution, and pipetted into cold 10% TCA. The precipitate was collected on a GF/A filter to determine "total cell-bound DNA." Another portion of the cell suspension was treated with 6 μg/ml DNase I at 37° for 5 min. After two washings, the suspension was pipetted into cold TCA and the precipitate was collected on a GF/A filter to determine DNA bound in a "DNase resistant" form. In two experiments (those illustrated in Figs. 1 and 3), cell-associated DNA was determined by using filtration onto a Gelman GA6 filter (pore size 0.45 μm) instead of using washing by centrifugation and TCA precipitation. In this case, 1 ml of the sample was filtered directly and washed with 0.15 M NaCl to determine "total DNA bound." In order to determine "DNase resistant DNA," 1-ml sample pretreated with DNase I was filtered as above. Control experiments showed that only small quantities (less than 0.05%) of either native or heat-denatured DNA are retained by the Gelman GA6 filters, provided that the filters have been presoaked in 0.15 M NaCl solution. (The numbers on the left and right ordinates should be multiplied by 10^{-4} and 10^3, respectively.)

We interpret the emergence of transformants as the completion of divalent-cation-requiring secondary steps of DNA uptake (and genetic transformation) by the preadsorbed molecules, penetrating the very same host cells onto which they have attached in the presence of EDTA. The correctness of this interpretation is indicated by the following three lines of evidence: (*i*) the rate of appearance (and yield) of transformants in the experiment illustrated in Fig. 3 is independent of dilution (evidence not shown); (*ii*) the appearance of transformants, nuclease-resistant and TCA-soluble DNA, and the loss of cell-bound DNA are not affected at all by the addition of 500-fold excess heterologous DNA to the medium (see Fig. 3). (*iii*) An alternative explanation for the emergence of transformants in Fig. 3 would be that upon removal of EDTA the adsorbed DNA molecules fall off the cells and then, subsequently, reattach to *any* competent bacteria and reinitiate a normal transformation process. We performed a direct experimental test of this possibility and obtained negative results. Competent sulfanylamide-resistant (SA^r) pneumococci were loaded in the presence of EDTA with DNA carrying the streptomycin resistance (Strep^r) marker. Upon resuspending the cell–DNA complexes in the medium containing Ca and Mg, we also added a suspension of competent micrococcin-resistant (K^r) pneumococci, and then performed the experiment exactly as in Fig. 3, except that the emergence of streptomycin-resistant (Strep^r) transformants of both bacterial populations was followed (by plating in media containing sulfanylamide plus streptomycin and micrococcin plus streptomycin). If the streptomycin-resistant transformants were caused by DNA molecules that have *first* detached from the carrier SA^r cells and *then* reinitiated the entire process of genetic transformation, one would expect that all competent pneumococci (including the K^r cells) would have equal chance for participating in such a process and thus the rate of emergence of the SA^{r}Strep^r and K^{r}Strep^r cells would be comparable. This is clearly *not* the case: it can be seen from the data in

TABLE 1. *Effect of experimental conditions on DNA binding and uptake by competent cells*

Experimental condition	Total DNA bound (cpm/ml)%	DNase-resistant %	DNase-sensitive %	Number of transformants per ml
Exp. 1. Strain R6				
C medium*				
+ albumin (control)	(839) 100	81	19	9.8×10^4
+ KCN (10^{-2} M)	(512) 100	46	54	2.4×10^4
+ NaN_3 (10^{-2} M)	(453) 100	42	58	3.6×10^4
+ iodoacetamide (10^{-3} M)	(187) 100	40	60	<300
+ *N*-ethylmaleimide (10 μg/ml)	(167) 100	32	68	<300
Exp. 2. Strain R6				
C medium				
+ albumin (control)	(881) 100	90	10	3.9×10^6
+ antimycin A (10^{-4} M)	(347) 100	30	70	1.1×10^5
+ oligomycin (40 μg/ml)	(253) 100	41	59	2.8×10^5
Exp. 3. Strain R6				
C medium				
+ albumin (control)	(562) 100	90	10	2.0×10^6
+ EDTA (10 mM)	(2013) 100	4	96	<300
SPA	(478) 100	86	14	2.5×10^5
without sucrose	(408) 100	73	27	4.2×10^4
without Mg and Ca	(504) 100	60	40	3.6×10^4
without Mg and Ca + EDTA (10 mM)	(2480) 100	3	97	<300
without Mg and Ca + EGTA (10 mM)	(2013) 100	4	96	<300
Exp. 4. Strain R6				
SPA (control)	(38203) 100	70	30	1.1×10^6
without Mg and Ca + murexide	(59805) 100	3	97	<300
without Mg and Ca + 2,6-dipicolinic acid	(56263) 100	3	97	<300

* C medium: ref. 4.

Fig. 4 that transformants of the K^r bacteria appear slowly and only after a lag of about 10–15 min, while a maximum number of transformants of the SA^r cells develop by the fifth min of incubation. The source of transforming DNA molecules for the K^r cells must be the DNA released to the medium from the competent SA^r cells.

A control experiment in which the genetic markers of the two cell types were reversed yielded identical results.

The Nature of the DNA Released from the Cells upon Readdition of Divalent Cations. Fig. 2 shows that the DNA molecules released to the medium had greatly reduced molecular weights,

TABLE 2. *Stimulation of cellular DNA binding by EDTA in competent cells*

Recipient	Donor [^{3}H]DNA	Total cell-bound DNA (cpm/ml) −EDTA	Total cell-bound DNA (cpm/ml) +EDTA
Cells grown to competence	Native	9,711	59,595
Incompetent cells		228	212
Incompetent cells + activator	Native	8,987	49,710
Incompetent cells + activator + trypsin	Native	374	328
Incompetent cells + activator in leucine-deficient medium	Native	253	316
Genetically incompetent cells (RA7)	Native	378	258
Cells grown in ethanolamine-containing medium	Native	478	480
Competent cells inactivated by heat treatment (Δ65°, 5 min)	Native	631	520
Competent cells inactivated by formalin-treatment (0, 5%; 30°, 5 min)	Native	358	393
Competent cells	Native	7,905	87,721
Competent cells	Native + excess (10×) nonradioactive homologous DNA	913	5,020
Competent cells	Heat-denatured	1,364	3,864
Competent cells	DNase-pretreated	196	316

but banded at or very near to the density of native DNA molecules. When the released DNA was used in a second round of transformation, we found that the intrinsic transforming activity (i.e., number of transformants per the amount of [^{3}H]DNA bound) was reduced by a factor of 3–5 as compared to the activity of the donor DNA used in the same experiment and at similar DNA concentrations.

Further Characterization of the DNA Attached to Competent Cells in the Presence of EDTA. Cells loaded with transforming DNA (in the presence of EDTA) were exposed to polycations (poly L-Ornithin and poly L-Lysine at 10 μg/ml), antiserum against double-stranded DNA, and mechanical shear (on a Vortex mixer) just prior to the readdition of divalent cations. All these treatments have reduced by 95–99% the number of transformants, while the same treatments applied to DNA-loaded cells *after* a brief (10 min) incubation in medium containing Ca, Mg, and sugar had no effect at all on the eventual number of transformants.

The Energy-Dependent State. Comparison of data in Fig. 3 and Table 1 suggests that a further resolution of the divalent-cation-requiring process(es) may be possible: it can be seen that release of cell-associated DNA can occur in a medium containing Ca and Mg but lacking glucose, while omission of sugar severely inhibits the appearance of genetic transformants. Competent bacteria were loaded with DNA carrying the Strep^r marker in the presence of EDTA. After removal of excess DNA, divalent cations were added back to the cells *without* the addition of glucose, and the suspension was incubated at 30°. After 10-min incubation in this sugar-free medium, glucose was added back to the cell suspension. During the first 5 min of the experiment, transformants appeared rapidly and did not increase any further between the fifth and tenth min of incubation. Upon the addition of sugar at the tenth min, the accumulation of transformants resumed immediately (yielding by the twentieth 1 min of the experiment a roughly 10-fold further increase). In an experiment of similar design, competent cells were loaded with radioactive DNA in the presence of EDTA and in the absence of sugar. The cell–DNA complexes were next transferred to two kinds of media: one with glucose, the other without glucose, but also containing the inhibitor 2-deoxyglucose; both media contained Ca and Mg ions. Table 3 shows that in the absence of glucose the amount of DNA converted to nuclease-resistance and the number of transformants was only 20% and 30% of that of the control values. On the other hand, the amount of DNA converted to TCA-soluble form and amount of DNA lost to the medium were unaffected by the absence of glucose.

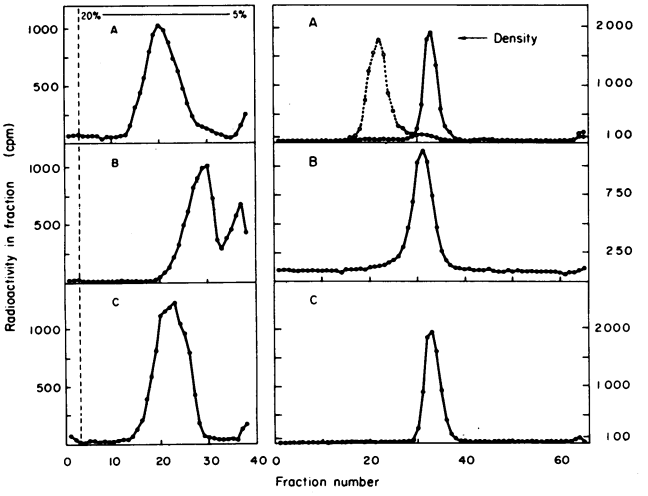


FIG. 2. *CsCl and sucrose gradient analysis of DNA loaded to cells in the presence of EDTA, and DNA released to the medium from "loaded cells" upon readdition of divalent cations.* A culture of competent cells was centrifuged and resuspended in SPA containing 10 mM EDTA at a cell concentration of 1.4×10^8 viable units per ml. After incubation with donor [^{3}H]DNA at 30° for 15 min, cells were centrifuged and washed twice by gentle resuspension in cold SPA lacking Mg^{++}, Ca^{++}, and EDTA to remove unbound DNA. Low speed centrifugation ($5000 \times g$, 7 min) in flat bottom polypropylene tubes was used to minimize shear. Under these conditions practically all the DNA attached to cells accumulated in a DNase-sensitive and shear-sensitive form (see *Results*). In spite of efforts to keep shearing forces to a minimum, substantial portion of cell-bound DNA was lost to the medium during centrifugation and thus the amount of cell-bound DNA represents a minimum estimate. The bacteria loaded with DNA ("DNA-loaded cells") were lysed by addition of 0.5% sodium deoxycholate in the presence of 10 mM EDTA. These extracts were deproteinized by shaking with an equal volume of chloroform (twice) and then dialysed against saline containing 0.01 M Na-citrate prior to characterization of the reisolated DNA. *CsCl and sucrose gradients.* Alkaline CsCl solutions (pH 11.0) were prepared by combining 0.6 ml of samples with 2.4 ml of a stock CsCl solution and by overlayering with 2 ml of mineral oil. The mixtures in polyallomer tubes (Beckman) were centrifuged at 37,000 rpm for 50–60 hr in a SW 50.1 rotor (Beckman) at 12°. Fractions of four drops were collected onto Whatman GF/A filter discs through a pinhole punched in the tube bottom. Centrifugation in neutral sucrose gradient was carried out using 5–20% linear gradients containing 1.0 M NaCl. A cushion of 60% sucrose was placed at the bottom of the gradient. The gradients, in polyallomer tubes, were overlayered with 0.2-ml samples and centrifuged at 37,000 rpm for 180 min in the SW 50.1 rotor at 20°. Fractions of eight drops were collected as above. Radioactivity was counted (with or without prior treatment of the samples with cold 10% TCA) in toluene scintillator by a Nuclear Chicago (Mark II) liquid scintillation spectrometer. Over 95% of the input DNA was recovered from the gradients. In the experiment shown in Fig. 2, [^{3}H]DNA released to the medium upon readdition of Ca and Mg to "DNA loaded cells" was collected by centrifugation after 10 min incubation at 30° with the divalent cations. Denatured DNA was prepared by heating for 10 min at 100° and chilling quickly. *Left:* sucrose gradients, *right:* CsCl gradients. (*A*) Pneumococcal DNA (*empty circles* in CsCl gradient represent heat-denatured pneumococcal DNA). (*B*) DNA released from "DNA loaded cells" upon addition of Ca and Mg. (*C*) DNA reisolated from "DNA loaded cells."

DISCUSSION

The Mechanism of EDTA Effect. The brief EDTA treatments used have no effect on cellular viability and there is no release of choline-containing surface polysaccharides (11). On the other hand, our experiments clearly show that the effects of EDTA (i.e., stimulation of DNA binding and inhibition of transformation) are caused by the removal of divalent cations from the system. Several of our observations described in this paper indicate that this removal of divalent cations affects the transformation process in part via inhibition of the Mg-dependent pneumococcal nucleases (12). At several points, our findings confirm and further develop recent observations of Morrison and Guild (2) and Lacks and Greenberg (3) con-

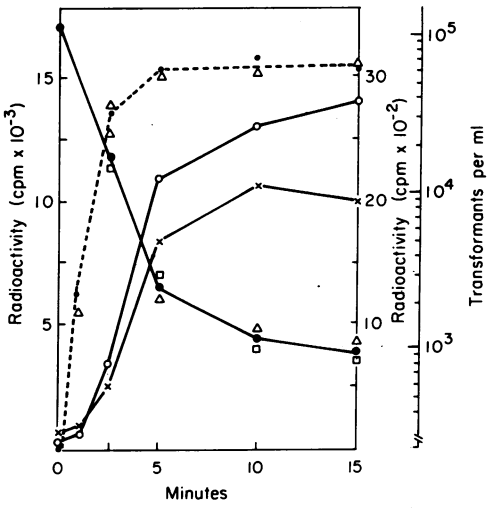


FIG. 3. *Removal of adsorbed DNA from the attachment sites upon readdition of divalent cations and energy source.* Competent cells were loaded with [^{3}H]DNA (in the presence of EDTA) at 30° for 15 min. After washing with EDTA-free SPA lacking Mg and Ca, "DNA loaded cells" received the missing divalent cations (0.3 mM Ca, 1 mM Mg) at 0 min, and were incubated at 30°. At frequent times, samples were tested for total and nuclease-resistant fractions of cell-bound DNA, cold TCA-soluble fragments in the medium, and for transformants. (●- - -●) transformants; (△- - -△) transformants in the presence of calf-thymus DNA added at 0 min together with the divalent cations; (●——●) total DNA retained by cells; (□——□) total DNA retained by cells incubated without carbon source; (△——△) total DNA retained by cells in the presence of calf-thymus DNA added at 0 min together with the divalent cations. Values for these curves are represented on the right-side ordinates. (×——×) DNA taken up in a nuclease resistant form; (○——○) TCA soluble fragments in the medium. Values for these latter two curves are represented on the left-side ordinate. (The numbers on the left ordinates should be multiplied by 10^{-3} and 10^{-2}, respectively.)

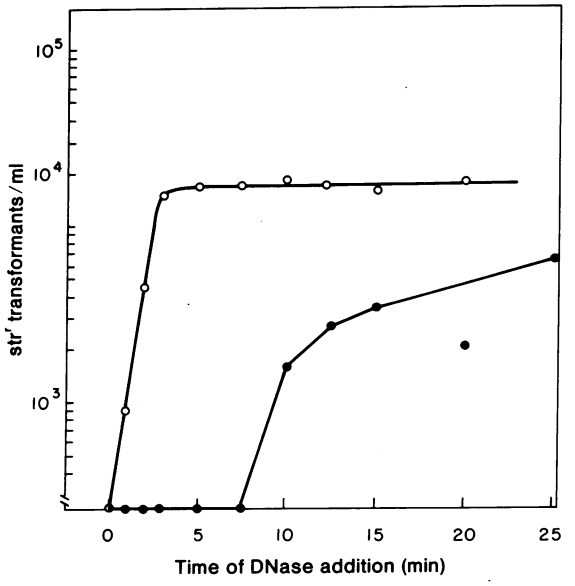


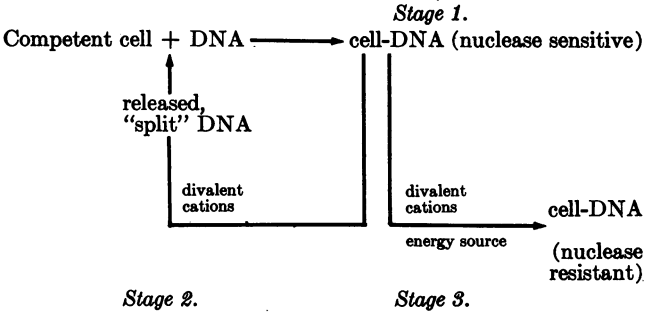
FIG. 4. *Completion of transformation process in DNA-loaded cells.* Competent SA^r pneumococci were loaded (in the presence of EDTA) with DNA carrying Strep^r marker. After removal of excess nonadsorbed DNA, "DNA loaded cells" were mixed with a suspension of competent K^r pneumococci of equal cell concentration. The mixed cells suspension (of DNA-loaded SA^r cells and nonpreloaded K^r cells) received Ca and Mg at 0 min and was incubated at 30°. Samples were tested for the emergence of Strep^r transformants of both bacterial populations. (○) Strep^r-transformed sulfanylamide resistant cells; (●) Strep^r-transformed micrococcin resistant cells.

cerning the role of divalent cations and nucleases in the genetic transformation of pneumococci. Morrison and Guild have shown that immediately or very shortly upon attachment to pneumococci DNA, molecules undergo endonucleolytic scission even before reaching the nuclease-resistant state (2). A similar endonucleolytic degradation of donor DNA molecules was first described by Dubnau and Cirigliano (13) in the transformation system of *Bacillus subtilis*. Our results confirm and further develop these observations: (*i*) in the presence of EDTA one can demonstrate an earlier step in the association of DNA molecules and cells from which intact molecules may be reisolated—this step may be analogous to the "EDTA resistant binding" of DNA observed by Morrison in *B. subtilis* (14) and to the shear-sensitive state of DNA molecules associated with *B. subtilis* cells (15, 16); (*ii*) the rapid endonucleolytic scission of the preadsorbed molecules can be activated as a second step following attachment of the DNA molecules to the competence-specific sites; and (*iii*) the addition of divalent cations induces a massive and very rapid release of the cell-bound (and initially intact) DNA into the medium in a fragmented form. These observations suggest that the loss of DNA from cell to outside is *caused* by the action of nucleases. While the released molecules have a smaller molecular size, nevertheless they retain their ability to reattach to competent cells and cause transformation, although with a lower specific activity. Such a sequential attachment → nuclease damage → and release seems to occur even under the conditions of conventional transformation assay (i.e., in competent cells that have not been exposed to EDTA) since molecules of lowered molecular weight can be detected in the supernatants of competent bacteria exposed to DNA (unpublished observations). It seems likely that during trans-

TABLE 3. *Requirements for conversion of donor DNA from nuclease-sensitive to nuclease-resistant form*

	Radioactivity (cpm)			
	Total	DNase resistant	Acid-soluble fragments	Transformants per ml
Initial DNA-cell complex	19,010	234	—	<300
After 10 min in:				
+ Mg and Ca + glucose	5,810	1,632	2,019	3.0×10^4
+ Mg and Ca − glucose (+ 2-deoxyglucose)	5,280	431	2,208	1.0×10^4
− Mg and Ca + glucose (+ EDTA)	13,860	225	18	<300
− Mg and Ca − glucose (+ 2-deoxyglucose) (+ EDTA)	12,101	224	81	<300

formation (in the absence of EDTA) competent cells rapidly "recycle" DNA molecules in a process catalyzed by surface-located endonucleases (the activity of which limits the amount of DNA retainable on the cell surface). By inhibiting the nucleases, EDTA "freezes" this dynamic system and one can resolve the forward (attachment) process from the nuclease-catalyzed release process, in time. We suggest that such a cyclic process (rather than a reversible association–dissociation of cells and DNA molecules) (7) may be the more realistic representation of the interaction between cells and DNA molecules.



(*iv*) Lacks and Greenberg have described the release of a fraction of donor DNA in the form of TCA-soluble fragments during transformation; the time course of release and its nutritional requirements (for energy source and divalent cations) were the same as those for nuclease-resistant binding of DNA (3). In our experiments the rate and extent of production of TCA-soluble DNA fragments was not influenced by energy deprivation (omission of glucose, addition of 2-deoxyglucose or antimycin A) even though transformation and nuclease-resistant binding of DNA were significantly inhibited (80% inhibition).

It is quite clear from the experimental evidence available that donor DNA molecules are exposed to extensive exonucleolytic and endonucleolytic activity during their attachment and penetration of the cell surface of competent pneumococci. Whether this nucleolytic activity on the donor molecules is essential for some phase of DNA uptake or simply reflects the hazards these molecules face while passing into the cellular interior, is still not clear at the present time.

It seems that even the earliest stage of transformation, i.e., the attachment of DNA molecules, requires *all* phases of activation, since cells treated with activator in the absence of protein synthesis or in the absence of choline would not bind DNA. This finding suggests that the multistep surface alterations initiated by the activator represent an "unmasking" process (such as a limited autolysin action localized at the cell-wall growth zone) which would expose DNA binding sites in a deeper layer of the cell surface, e.g., on the plasma membrane not normally accessible from the outside. It is interesting to consider the possibility that these binding sites might be surface-located nuclease molecules.

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