[8] The term "freezing of the particles" is used for convenience. As it is uncertain whether or not there is an alteration in the physical state of the particles when frozen at −20°C, the term is therefore descriptive of the state of the suspending medium.

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*ON THE ROLE OF INTEGRITY OF DNA PARTICLES IN GENETIC RECOMBINATION DURING PNEUMOCOCCAL TRANSFORMATION**

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The bacterial transformations are especially appropriate for studying molecular events involved in genetic recombination because the two components, cells and chemically purified DNA, may be modified independently before entering into the reaction. In the experiments reported here, a three-marker linkage group bearing streptomycin and sulfonamide resistance in Pneumococcus is used to evaluate the structural requirements for the marked regions of DNA to undergo recombination with recipient cells. A kinetic technique has been developed[1] which serves to demonstrate and quantitate genetic linkage in a three-factor cross.

With this system, recombination frequencies have been examined as a function of the state of the DNA or of the cells. Localized changes of primary structure in thermally degraded DNA are interpreted to increase the frequency of genetic recombination at or near those sites. On the other hand, collapse of secondary structure of DNA by thermal denaturation[2-4] has a relatively minor effect on linkage frequency in DNA preparations of good quality, even though the biological activity of such samples is reduced by over 95% and those particles retaining activity are themselves denatured.[4] Since recombination frequencies depend on the temperature at which competent cells are exposed to DNA,[5] the recombination seems to be related to metabolic functions of the cell.

Materials and Methods.—Experiments were performed with the wild-type R_1 (+++) strain of *Diplococcus pneumoniae*, a single colony derivative of R_6, and with drug-resistant variants to streptomycin (*S*), micrococcin (*K*), and sulfonamide (*a*+, *d*, *ad*) obtained as previously described.[6] The viable unit averages four discrete cells on the basis of microscopic counts in a Petroff-Hausser chamber. DNA was prepared by the chloroform-isoamyl alcohol method[7] with 3–6 deproteinization cycles for a given preparation. The native DNA content of a purified sample was estimated from the degree of hyperchromicity at 260 mμ,[8] and the quality of the preparation judged by comparing its maximal biological yield to that of other well-characterized preparations.[9]

For transformation, cells were grown to competence in supplemented enzymatic casein hydrolysate medium (CH),[6] synchronized,[10] and frozen in glycerol as previously described.[11] Thawed at 0°C immediately before use, cells were concentrated after collection on Millipore filters of porosity 0.45 μ. After resuspension in CH medium supplemented with Ca^{++} and bovine serum albumin,[7]

the cells were preincubated for 15 min at 30°C before addition of DNA at zero time. At intervals thereafter, aliquots of the transformation mixture were removed to two volumes of meat-infusion broth[5] containing 75 μg/ml of deoxyribonuclease (Worthington 1× recrystallized) to destroy active DNA in solution. After 90 min at 37°C to allow phenotypic expression, the transformants were selectively scored in the presence of appropriate drugs in liquid medium and antibody.[9] The selective scoring conditions were: *S*—150 μg/ml dihydrostreptomycin sulfate/ml; *K*—100 mμg micrococcin/ml; for the different classes of sulfonamide resistance, *a*+ in 15 μg p-amino-salicyclic acid/ml and 40 μg *p*-nitrobenzoic acid/ml, +*d* and *ad* (referred to as *d*) in 50 μg sulfanilamide/ml, and *ad* in 250 μg sulfanilamide/ml. The sulfonamide resistance markers form part of a complex locus to which the *S* marker is linked in the unambiguous order *Sad*.[5] The *K* marker is unlinked to the other three markers.

Modification of DNA was performed by the methods of Roger and Hotchkiss.[4] For subcritical heat inactivation, DNA samples at 10 μg/ml in 0.14 *M* sodium chloride and 0.02 *M* phosphate buffered at pH 6.8 were heated at 85°C, followed by rapid chilling of treated aliquots. Critical heat inactivation was performed on aliquots diluted to 25 μg/ml in the same solvent and heated for 15 min at the supracritical temperature of 95.5°C; for denaturation one aliquot was rapidly chilled in a pre-iced tube, and the other was renatured by cooling over about two hr to 25°C.

The linear DNA concentration dependence of transformation yield in an otherwise constant system[9,11,12] suggests that only one DNA particle is required to initiate irreversible transformation to one genetic marker. We have shown[1] that the linear accumulation of transformants (either singly-[11] or multiply-marked) with duration of exposure to DNA may be similarly used to demonstrate that genetic linkage is due to the coexistence of several markers within one DNA particle. Linkage frequency is defined as the ratio of the linear rates of accumulation of double-to-single transformant classes. Two unlinked markers, such as *S* and *K*, accumulate quadratically in time at a frequency very close to that calculated on the basis of independent, successive interactions of one cell with the two DNA particles.[1]

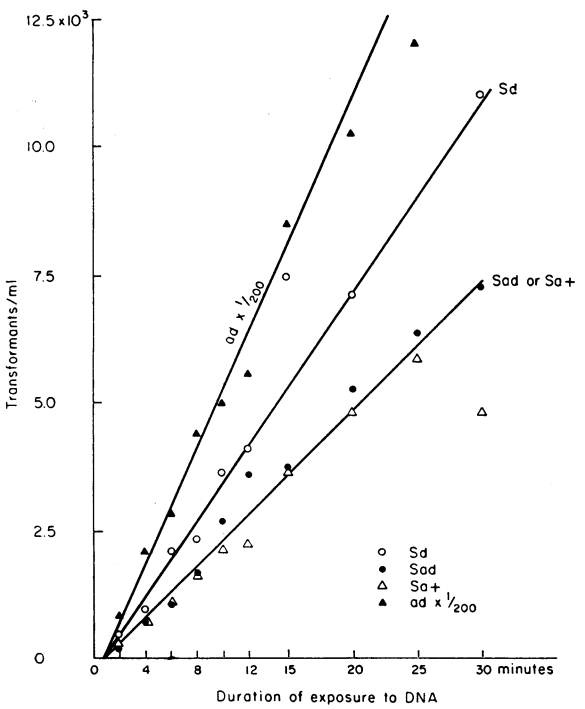

FIG. 1.—The linear accumulation with time of multiply-marked transformants to linked markers. A concentrated, competent (+++) culture at 4 × 10^7 viable count/ml was exposed to 0.29 μg *Sad*-DNA/ml at 30°C. The markers are: *S* = streptomycin resistance; *a*+, *d*, *ad* = different and phenotypically distinguishable markers for resistance to sulfonamide.

Experimental Results.—Three-factor crosses: When a wild-type (+++) recipient is transformed by an *Sad*-DNA, all of the transformant classes, as indicated above, accumulate linearly[1] from the time of addition of DNA (Fig. 1). The rate of accumulation of the *ad* class is much greater than for the *Sd* class defining, respectively, 50 per cent and about 3 per cent linkage relative to *d*. Both the *Sd* and the *Sad* categories accumulate linearly, which suggests that the *S*+*d* class is also appearing as a result of transformations initiated by single particles marked *Sad;* this recombinant type, as estimated from the differential linear rate, constitutes about

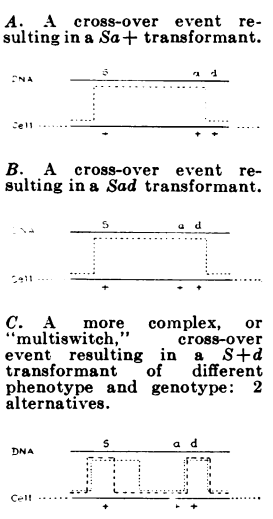

FIG. 2.—Diagrammatic representation of "switches" occurring during genetic recombination.

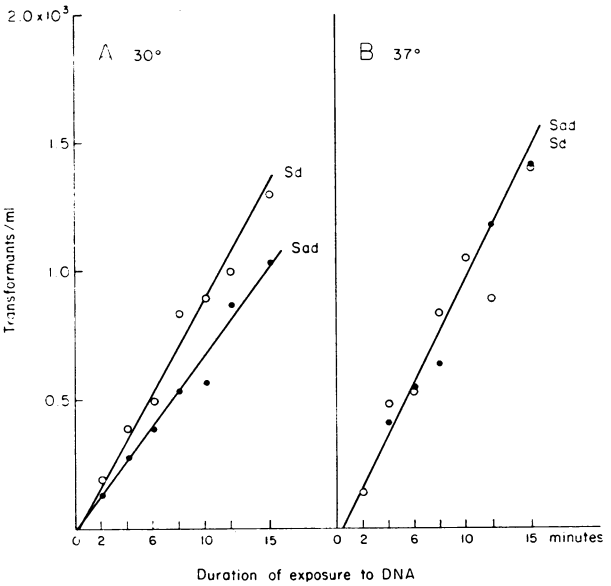

FIG. 3.—Temperature dependence of proportion of complex to simpler recombinations. (+++) culture was diluted to 1 × 10^6 viable count/ml in medium at 30°C (*A*) or 37°C (*B*), containing 0.5 μg *Sad*-DNA/ml. Markers described in legend to Fig. 1.

40% of all *Sd* transformants. This *S*+*d* genotype would result from a multiswitch recombinational event as diagrammed in Figure 2. Entirely parallel results were obtained for a different distribution of the same markers, in which the multiswitch transformant could be directly scored.[1] Equivalent exchanges have recently been reported for the *B. subtilis* transformation system by Nester *et al.*[13] The data on the pneumococcal system have been shown to reflect (single) genetic events within one division cycle rather than selection artifacts or segregation phenomena.[1]

Temperature dependence: When two aliquots of a freshly-grown competent (+++) culture are exposed to *Sad*-DNA either at 30°C or at 37°C, the linkage frequencies of *Sa*/*S* and of *Sd*/*S* are not affected significantly, but the multiswitch intramolecular events effectively disappear at 37°C, in that the yield of *Sad* now accounts very nearly for the total *Sd* yield (Fig. 3). Since the more complex events can be eliminated by changing the state of the cell population without altering that of the DNA preparation, it becomes impossible to attribute their occurrence exclusively to the particular physical state of a given DNA preparation. To interpret data obtained with degraded DNA samples, it is important to recognize this active role played by the cells.

Subcritical heat inactivation: Assay of DNA samples which have been subjected to increasing degradation at subcritical temperatures reveals initial multihit damage followed by exponential inactivation for all single markers[4] and groups of markers (Figs. 4 and 5). The ultimate exponential rate of inactivation is related to the number of markers assayed in the transformants, but is independent of their genetic (and physical) relationship to one another. The single marker transformations to *d* and *a*+ are inactivated at an exponential rate only slightly slower than the single

S marker, but the logarithmic decay rate for all double-marker transformations is twice as great, whether these be for the unlinked pair *SK*, the loosely-linked pairs *Sa*+ and *Sd*, or the tightly-linked pair *ad;* the triple transformations to *Sad* are inactivated at very nearly three times the exponential rate of the constituent single markers. Fox has observed similar differential inactivation of some marker

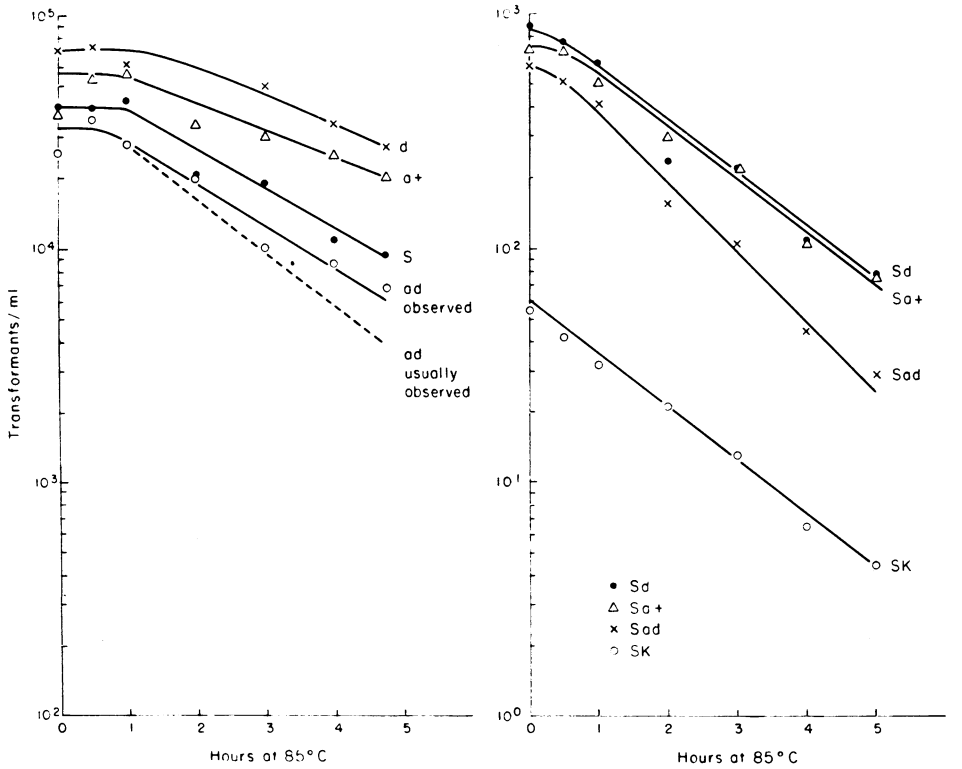

FIG. 4.—Subcritical heat inactivation of a multiply-marked *SadK*-DNA. Effects of thermal treatment denoted in abscissa were bioassayed on a concentrated, wild-type culture at 3×10^7 viable count/ml exposed to 0.02 μg *SadK*-DNA for 5 min at 30°C; this is in the linear response range. Markers are described under Fig. 1 with *K* as unlinked marker to micrococcin resistance.

groups concomitant with P^{32} decay in labeled pneumococcal DNA.[14] For the transformation experiment of Figure 5, in which the *a* allele is present in the recipient strain, equivalent rapid inactivation of multiple marker groups is obtained. This means that the ratio of *Sad/Sd* and of *ad/d* decreases with heating for the experiment of Figure 4 but increases in that of Figure 5. Although the linkage order is *Sad*, the inactivation of the *Sd* pair or its integration into the genome during transformation is apparently independent of the presence of a central *a* region.

Critical heat denaturation and renaturation: The rapid critical inactivation of pneumococcal-transforming DNA at higher temperatures is accompanied by denaturation of the marker particles. The altered DNA has lower activity than native material even at saturating concentrations and cannot compete effectively with the latter, so that its small activity is not due to a residual content of native material.[4] On suitable subsequent treatment at intermediate temperatures, however, the de-

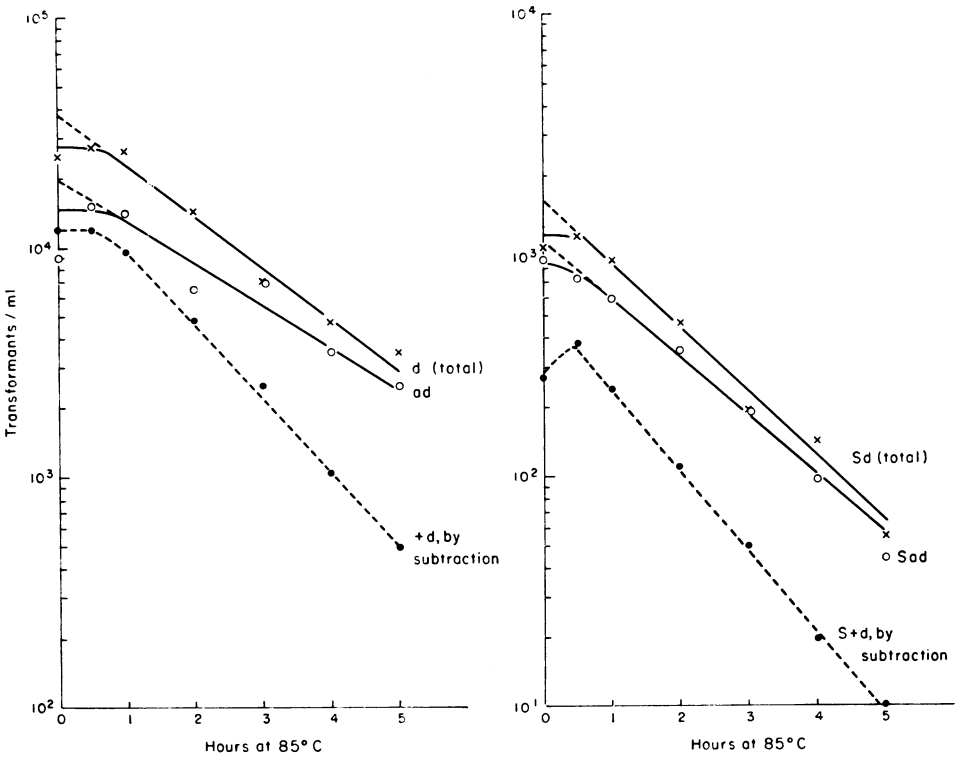

FIG. 5.—Subcritical heat inactivation of a multiply-marked $S+d$-DNA. Experimental conditions are described in legend to Fig. 4. The recipient culture is (+a+), slightly resistant to sulfanilamide; note that *ad* represents a transformant to a single marker (*d*), since the *a* allele is present in the recipient. The exponential rate of inactivation is unusually rapid.

natured sample can be partially renatured concomitant with a partial return of the secondary structure toward that of the native state.[2, 3]

Extensive kinetic experiments on many independently denatured aliquots of several DNA preparations have shown that genetic linkage is not destroyed by denaturation. For example, 50 per cent *ad/d* linkage may be completely preserved[4] (Table 1). The more loosely linked pairs of markers involving streptomycin and

TABLE 1

LINKAGE FREQUENCIES IN DENATURED AND RENATURED *Sad*-DNA

Heating experiment	% *d* survival	*ad/d*	*Sd/S*	*Sad/S*	*Sa+/S*	*Sad/Sd*
			Unheated standard			
0	100	0.56	0.079	0.057	0.028	0.72
			Residual activity of denatured DNA			
1	3	0.41	0.02	0.01	0.008	0.5
2	4	0.55	0.02	0.01	0.01	0.5
3	1.2	0.56	0.048	0.020	0.020	0.42
			Activity of same samples, renatured			
1	46	0.29	0.02	0.01	0.012	0.5
2	33	0.23	0.012	0.006	0.009	0.5

Assays were performed on (+++) culture at 4×10^7 viable count/ml exposed to 0.2 μg DNA/ml at 30°C. Each rate is based on 4–10 time points. Each experiment was performed after heating separate aliquots of the same DNA preparation. Experiment 0 represents a control in which the DNA has not been denatured. A ratio of less than unity for *Sad/Sd* indicates multiswitch recombinants ($S + d$).

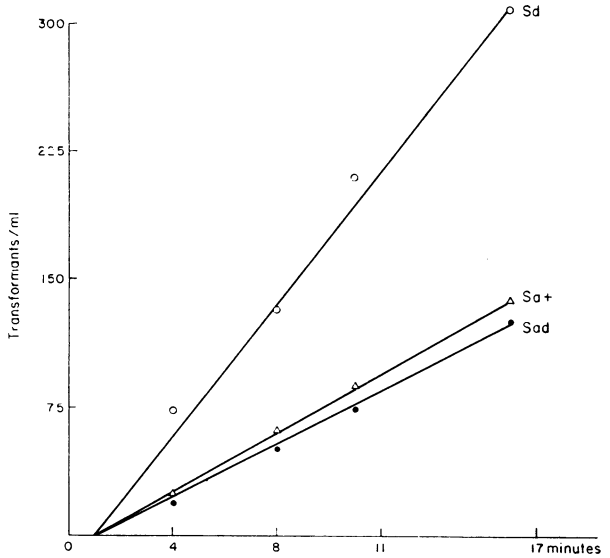

FIG. 6.—The linear accumulation of multiply-marked transformants from a heat-denatured DNA preparation. Data are taken from the experiment summarized in Table 1, heating 3; linkage between streptomycin and sulfonamide resistance is about 50% that of the unheated sample.

sulfonamide resistance survive denaturation imperfectly and less reproducibly, but the linkage remains clearly demonstrable (Fig. 6). The multiswitch recombinational events persist at a relative frequency near or slightly greater than for unheated samples. When renatured aliquots of these denatured samples are used in transformation, the linkage frequencies either remain unchanged or decrease (Table 1); linkage is not increased although renaturation restores the maximal activity for single markers to about 40% of the values for unheated samples. Multiswitch recombinational events still occur at near the control frequency. Thus, loss and partial restoration of secondary structure are not correlated with significant changes in linkage frequency.

Further loss of linkage on renaturation of some samples may be due to subcritical inactivation during the slow cooling from elevated temperatures. Brief exposure a few degrees below the critical temperature does not alter the biological activity of a sample, but the markers, upon rapid chilling, become more susceptible than in unheated material to prolonged exposure 25°C below the critical value (Table 2).

Annealed mixtures of genetically distinct DNA: After denaturation and renaturation of mixtures of genetically distinct DNA's, the time course of accumulation of multiply-marked transformants should indicate whether new species of biologically active DNA particles have been formed. This experiment, first suggested by Marmur and Lane,[2] had led to small increases in double marker incorporation in the Hemophilus transformation system.[15] For the pneumococcal system, Marmur was unable to detect the formation of any new linkage groups.[16] For a successfully

TABLE 2

EFFECT OF SLOW COOLING FROM ELEVATED TEMPERATURES ON BIOLOGICAL ACTIVITY

DNA	Originally heated 15 min at 87°C			Originally heated 15 min at 92.3°C		
	d per ml	*ad* per ml	*ad/d*	*d* per ml	*ad* per ml	*ad/d*
Unheated control	1.0×10^5	5.8×10^4	0.58	1.2×10^5	7.2×10^4	0.60
Heated and iced	9.4×10^4	4.5×10^4	0.48	1.8×10^3	1.0×10^3	0.56
Renatured, 65°C						
0.5 hr	7.0×10^4	3.9×10^4	0.56	9.1×10^3	3.1×10^3	0.34
1.5 hr	6.8×10^4	3.2×10^4	0.47	2.1×10^4	5.0×10^3	0.24
2.5 hr	6.4×10^4	3.2×10^4	0.50	2.8×10^4	7.7×10^3	0.27

The critical temperature for sulfonamide resistance markers is about 90°C under these conditions. Assays were performed by exposure of 6×10^7 viable count/ml to 0.84 μg DNA/ml at 30°C for 30 min. One hr exposure to 65°C of untreated material causes a negligible loss of biological activity (<10%).

renatured mixture of $S+d$ and $K...a$ DNA, the data show quantitatively that no new species are formed in significant frequency which simultaneously carry the previously separate markers a and d (Fig. 7); the quadratic pattern of accumulation of ad transformants is similar to that of SK (unlinked) and Sa (loosely linked if present within one genome) types. Nor are mixed clones of transformants recovered which contain each of the two cell types $a+$ and $+d$. Positive results in this experiment would have suggested the formation of genetically hybrid entities, which participate as such in recombination, and would have given further insight into the molecular events occurring during denaturation and renaturation of DNA.

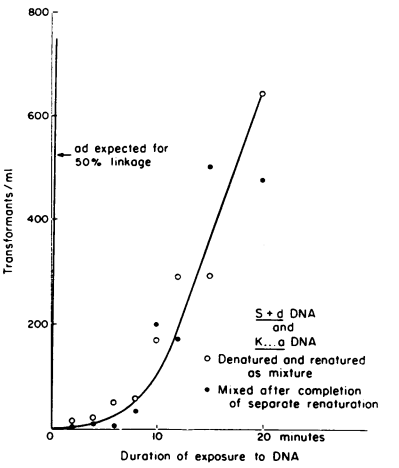

FIG. 7.—Time course of appearance of ad transformants after exposure to renatured mixtures of DNA ($S+d$ and $K \ldots a$). Experiment was performed at 30°C on wild-type cells at 4×10^7 viable count/ml and DNA at a total final concentration of 0.4 μg/ml (i.e., 0.2 μg/ml/DNA). The renaturation yield was 45% for both the S and K markers relative to the intact control mixture. The linkage of Sd/S dropped from 3.0% to 0.05% after denaturation and renaturation. The $+d$ transformant accumulated at a linear rate of 4.5×10^3/min.

Discussion and Summary.—Multiswitch intramolecular recombinational events in three-factor crosses between cells and DNA particles show little or no genetic interference, but their occurrence has been observed to depend on the temperature at which competent cells are exposed to DNA. They reflect cellular processes governing genetic recombination and do not arise from the state of a DNA preparation. The sensitivity to physiological conditions may create ambiguities that make genetic maps indeterminate or, as previously suggested,[5] distort them, but appropriate choice of physiological state permits unambiguous determination of map order.

Acid catalysis of slow subcritical inactivation of transforming DNA[4] and of purine release from DNA[17] has led to the suggestion of depurination as a mechanism of heat degradation.[4, 17] Whatever its nature, the lesion is clearly localized and submolecular because the rate of inactivation of biological activity depends strictly on the number of markers assayed whether or not they are in a linkage group. Subcritical heat inactivation seems to alter the proportion of complex recombinations as reflected by the ratio Sad/Sd recombinants (Figs. 4 and 5); this differential inactivation rate is another confirmation of the occurrence of multiswitch recombinations on the intramolecular level.

In addition, cells seem to be able to escape the consequences of exposure to heat-degraded DNA inasmuch as the genetic recombination occurs so as to bypass the inactivated region, thereby retaining the homologous segment of the host genome. Several interpretations of the differential inactivation rate of Sad and Sd types appear unlikely. Subcritical heating does not appear to have a mutagenic effect on dissolved transforming DNA at neutral pH[18,19]; nor can loss of function (due to incorporation of an inactive region) be invoked as a mechanism, since shift from wild-type to the a allele alters the catalytic properties of an essential protein but not the fact of its synthesis or nonsynthesis.[20, 21] Finally, no lethal effect has been

observed, inasmuch as there is no apparent loss of viability in competent pneumococcal populations when these are exposed to severely degraded DNA, although heat-degraded DNA penetrates rather readily into cells.[22, 23] The possibility of suicidal transformation, due to genetic incorporation of an inactivated region which, in the active state, controls an essential function, has not been rigorously excluded because of the chain-like growth of Pneumococcus (an average of four cells per colony-forming unit) and because of the complex growth medium which provides an unknown number of essential growth factors. If damaged regions of DNA were incorporated, one might expect either a mutagenic or a lethal effect, neither of which has been observed. It may very well be that the damaged regions of DNA particles are not integrated at all or are eliminated from the genome shortly after integration through an effect of the lesion. Either of the latter two alternatives is equivalent to saying that a polynucleotide strand bearing a heat-induced lesion will only be incorporated if there is an eventual "switch" at or near the site of damage. A "hole" in the template, as produced by a depurination, could then represent a localized signal for recombination.

The preservation of linkage in denatured and renatured DNA samples suggests, on the other hand, that an extended region of helical structure is not necessary for recombination. We attribute the variable and partial loss, in denatured aliquots, of the loose linkage between streptomycin and sulfonamide resistance to subtle pre-existing flaws in different preparations of DNA (perhaps single-strand breaks) and to uncontrollable factors in heating experiments. Such partial loss of linkage, when it occurs, is not restored by renaturation, although the single marker yield is thereby increased at least tenfold. The extent of helicity affects more the particle's ability to penetrate into a cell, a conception strongly supported by the observation that biological activity and P^{32}-fixation of labeled DNA are decreased to roughly the same extent by denaturation but to different extents by degradation.[22]

* This work was partially supported by grant AI-03170 from the National Institutes of Health and grant G-12894 from the National Science Foundation to the Rockefeller Institute (Dr. Rollin D. Hotchkiss, principal investigator).

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*CHLOROPLAST DNA IN CHLAMYDOMONAS**

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Extranuclear DNA has not been considered a normal cellular constituent either by cytologists or by geneticists, although exceptional instances have been recognized, such as the cytoplasmic DNA of the amphibian oöcyte.[1] Nonetheless, suggestive evidence has been reported of DNA in chloroplasts,[2, 6, 7] in mitochondria,[3] and in the kinetoplasts.[4]

The present study was prompted by two considerations, one genetical and the other biochemical. We have recently isolated a series of nonchromosomal mutants in the green alga *Chlamydomonas*, which indicate the presence of an extensive nonchromosomal genetic system.[5] Many of these strains cannot grow photosynthetically, suggesting that mutations have occurred in nonchromosomal genes which affect chloroplast traits. Where are these nonchromosomal genes located and are they composed of DNA? A recent report that *Chlamydomonas* contains extranuclear Feulgen-positive particles,[6] and that DNA isolated from *Chlamydomonas* contains a satellite band with a buoyant density lighter than that of the major component,[7] led us to examine the nucleic acid content of chloroplasts isolated from *Chlamydomonas*.

In this paper we describe a procedure for isolating relatively intact chloroplasts free of other cellular debris. DNA extracted from these isolated chloroplasts has been characterized with respect to buoyant density and base composition.

Materials and Methods.—(1) *Isolation of chloroplasts:* Cells (*Chlamydomonas reinhardi*, strain 21 gr) were grown with a six-hr doubling time in 10-liter batches in minimal medium with 5% CO_2,[8] harvested from the logarithmic phase, washed, and resuspended in W medium.[9] W medium contains 0.25 *M* sucrose, 2.5% Ficoll (Pharmacia), 5% dextran-40 (Pharmacia), 0.006 *M* mercaptoethanol, 0.01% bovine serum albumin, 1×10^{-3} *M* $MgCl_2$, 1×10^{-3} *M* $MnCl_2$, and 1.5×10^{-3} *M* $CaCl_2$ in 10^{-2} *M* tris-HCl buffer at pH 7.8. Cells were broken in a cold French pressure cell at 3,000 psi. All subsequent steps in the isolation procedure were carried out at 2°. Five ml of the broken cell mixture was layered onto a discontinuous gradient containing 5 ml each of 2.5 *M*, 2.0 *M*, 1.5 *M*, and 1.0 *M* sucrose in W medium. The gradients were centrifuged in the Spinco Model L at 25,000 rpm in the SW 25.1 rotor for 90 min.

After centrifugation five green bands were present, as shown in Figure 1. Examination in the light microscope revealed that bands 1 and 2 contained broken chloroplasts, band 3 contained largely intact chloroplasts, bands 4 and 5 contained chloroplasts and some unbroken cells. The bands were recovered by piercing the bottom of the tube and collecting drops. Only band 3 was retained for further purification, diluted with W medium not containing sucrose, and banded again in a sucrose density gradient as above. After centrifugation, band 3 was again collected