*MANIFESTATION OF LINEAR ORGANIZATION IN MOLECULES OF PNEUMOCOCCAL TRANSFORMING DNA**

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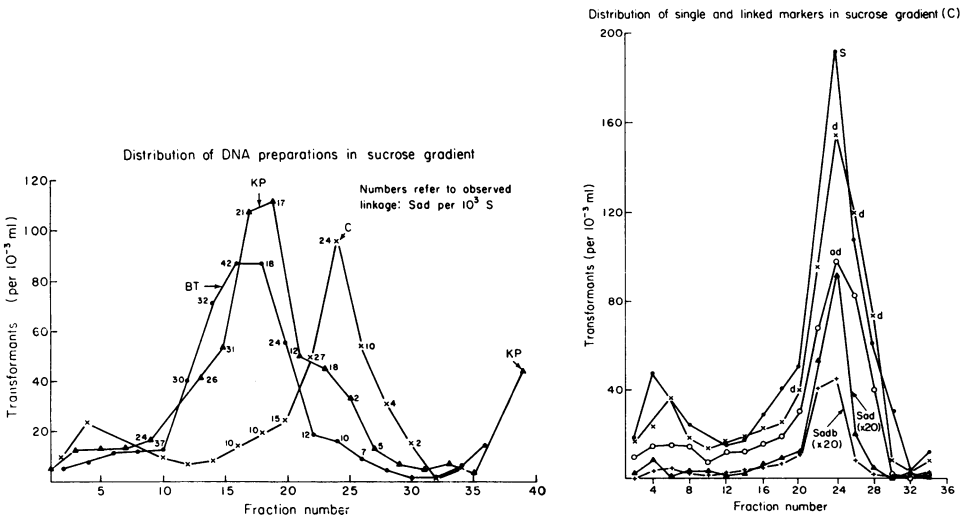
The DNA plus cell interaction leading to bacterial transformation is an orderly two-particle reaction, dependent upon the first power of cell and DNA concentration,[1-4] and of the duration of the extracellular contact.[3-5] When two physically unlinked markers are incorporated into the same cell, the double transformations become proportional to the second power of DNA concentration[4, 5] or duration of exposure.[5] The competitive power of other DNA[6] and the kinetics[3, 7] suggest an early reversible step, but DNA is eventually physically incorporated.[8-10] The DNA which enters pneumococcal cells is rapidly separated into two equal fractions, high-molecular and degraded,[11] and the regions associated with the introduced marker activity appear to be large tracts carrying only a single strand from the introduced donor DNA.[12] In *Hemophilus*[13] and *Bacillus subtilis*[14] cells also, it seems that only one transforming DNA strand survives intact.

The reproducibility of the maximal pneumococcal transforming activity for several markers, as well as of various linkages, in DNA prepared without deliberate shear, has suggested that the recovered material may have relatively regular points of rupture when separated from the presumably much longer bacterial chromosome. This could happen if there were fixed discontinuities or regions of relative lability in the chromosome. This report will show that there is a tendency for reproducible sequences of marker entry in transformation by pneumococcal DNA, suggesting that the molecules as isolated from the cells have to some degree uniform terminations. The study makes use of separated single strands of DNA, reactivated by complexing with unmarked homologous DNA.

Methods and Materials.—Deoxycholate lysates of pneumococci were used for all preparations of DNA. The preparation of chloroform gel DNA (C) and the methods of transformation have been frequently described.[3, 15] The marker strains were multiple mutants and transformants of wild type (R6, derived from R36A), and the competent strain was a single colony derivative (R1-26) of the same strain. Zone centrifugation was done with 1 μg DNA in 5–20 per cent sucrose gradients in saline at 34,000 rpm for 3 hr in the cold in celluloid tubes, and fractions of 2 drops collected from the bottom in sterile transformation medium and biologically tested. DNA preparations of type C were denatured with 0.1 *M* NaOH at 25°C, then neutralized at 0°C in concentrations of about 25 μg/ml and put on 1-cm columns containing 5 ml of MAK having 0.7 the quantity of methylated albumin previously specified.[16] About 0.2–0.6 mg of denatured DNA in 0.6 or 0.7 *M* NaCl was used; "A" fractions came at about 0.85 *M* and "B" at about 1.0 *M* salt. Renaturation was in 1.5 *M* NaCl at 65°C for 30 min, followed by 1–2 hr cooling; biological activity was measured at concentrations giving linear response. Rescue of denatured DNA was accomplished by renaturation with a double quantity of wild-type DNA and measured per unit of marked DNA present, expressed as per cent of activity given by native marked DNA.

Results and Discussion.—Studies with DNA isolated with minimal degradation: Berns and Thomas[17] prepared *Hemophilus* transforming DNA by phenol extraction and dialysis, and though the method does not remove polysaccharides, RNA fragments, etc., it avoids some questionable precipitation steps, produces DNA of increased sedimentation coefficients and viscosity, and higher linkage. Kelly and Pritchard[18] used cautious precipitations and manipulation of phenol-extracted *B. subtilis* DNA at high concentrations to produce partly purified DNA showing high cotransfer of marker pairs. Pneumococcal DNA preparation KP was made by a modified Kelly-Pritchard[18] method which included gentle precipitation with alcohol, rather than 2-ethoxyethanol. Preparation BT was by a method like the simplified[19] Berns-Thomas procedure, except that metaborate-washed phenol[18] was used, and one gentle alcohol precipitation after RNase treatment and dialysis to remove major contaminating ribonucleotides.

As shown in Figure 1, separate zone centrifugation in sucrose density gradients distributes these two and a standard chloroform gel (C) preparation with different sedimentation velocities in the order BT > KP > C. Most interesting are the relative "linkage activities" for markers *Sad*, indicated by the numbers along the graphs. It will be seen that the most rapidly sedimenting components of each preparation show highest linkage, but even in preparation C there is a portion giving higher linkage than the more rapidly sedimenting material at the trailing edge of the bands of the KP or BT preparations. This may mean that each preparation contains particles with a range of sizes and degrees of integrity which are not well separated in a sedimentation band. Consequently, a C preparation which also gave the sharp banding pattern of Figure 2 was chosen as the material to be molecularly "mapped." C preparations are easily made, stable, are free of major contaminants, except 10–20 per cent of ribonucleotide fragments, and not easily



FIGS. 1 (*left*) AND 2 (*right*).—Zone centrifugation of 1 μg DNA (in 0.1 ml) into linear sucrose gradients. Bioassay in concentration dependent range with standardized culture. Markers: streptomycin resistance (*S*), both alone and linked to sulfonamide resistance (*d*, *b*, and linked *ad*, *abd* factors[20]). DNA preparations *BT*, *KP*, and *C* described in text were run separately.

damaged by chromatographic and other manipulations, and furthermore are the standard preparations whose reproducibility has so often been noted. The preparation C showed maximal (plateau) activities for at least three markers, as high as those of all good preparations of this laboratory and, to the best of our knowledge, equal to or better than those of any other preparations made from this organism.

Rate of marker entry—double-stranded DNA: The results of Lacks[11] suggest that DNA enters cells in endwise fashion. If so, one or another marker should enter first in any given cell. Kent and Hotchkiss[5] found with both single and linked markers at 30°C linear rates for entry terminated by DNase. There was little lag before the first detected entry of each marker. In order to identify stages in the entry, a method was needed to distinguish marker DNA attached to the cell but not yet entered from that already entered or not yet attached. This was found in terminating the cell DNA reaction in two different ways: (1) nuclease interruption as usual by DNase which rapidly destroys all attached and unattached DNA still outside the cells; and (2) a swamping dilution into an excess of unmarked native DNA which reduces to 2–5 per cent all further initiation of marker DNA entry, while presumably allowing completion of entry of all molecules which had begun to enter. DNase is added here too after allowing some time for completion of entry. The number of transformants found in (1) correspond to markers in the already entered portions of DNA, and those found in (2) minus (1) are a measure of the number of markers in the extracellular portions of attached DNA at the same moment.

In this way, patterns of marker entry (Fig. 3) were obtained which suggested regularities in the rates at which markers became available to transforming cells. For 7 min after a swamping dilution into unmarked DNA, addition of DNase could destroy markers in the order $b > d > S$, which is also the linkage order reported for

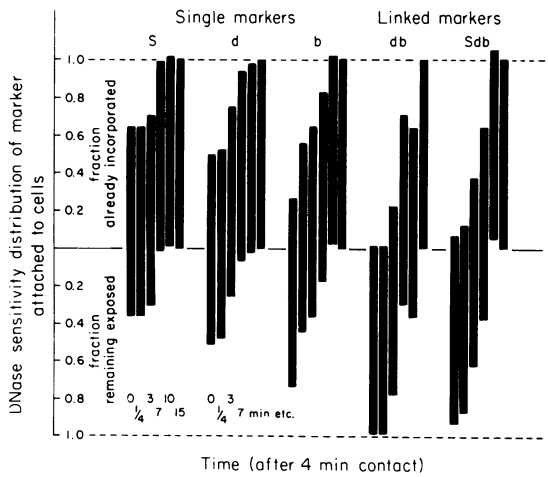


FIG. 3.—Transformants obtained from cells exposed to native DNA (*Sdb*) for 4 min, then swamped with excess of unmarked DNA. Extent of subsequent arrival of DNase-insensitive state (cell entry) by different markers, expressed as fraction of the total which achieves insensitivity by 15 min (which represents total attached at the 4-min time). DNase addition at 0, ¼, 3 min, etc., after swamping; the upward progression of the bars reflects the average movement of the marker into the cells at that time. Markers as designated in Fig. 1.

these markers.[20] This suggests that they penetrate into transforming cells in the order $S > d > b$ as a linked array, or at least that populations of molecules deliver them in that order.

At this point in our work we had generous access to data of Levine and Strauss[21, 22] who detected a time difference for entry of pairs of linked markers into *B. subtilis* cells, and short lag periods before individual markers entered, although without evidences of polarity. They also noted a greater transfer, without lag, if transformation was terminated by simple dilution. The longer interval required for entry of linked markers is also seen in Figure 3. This has been interpreted[22] as indicating the linear mode of entry of the linkage group, without reference to polarity. The semilogarithmic plot used by Strauss to estimate lag in entry tends to emphasize this lag but is very sensitive to the earliest low yields. Although the *B. subtilis* transformations do not appear to follow a linear time course as the pneumococcal ones do,[5] multiple-particle transformations which are likely to contribute to double marker incorporation would exaggerate an apparent logarithmic "lag" in entry. Our own data do not exclude this possibility, for two-step transformations could possibly give the patterns shown by *bd* and *Sbd* accumulations. Strauss has recently[23] considered two-particle transformations as an explanation and also new evidences that populations of transforming particles may contain different marker distributions. The more rapid processes of uptake by *Hemophilus* cells[24] have not revealed marker arrays.

The observations already cited[11, 12] and the failure to create linkage by annealing two differently marked DNA's,[25–27] even in a quantitatively very favorable case,[26] indicate that only one strand of native DNA is used, although it may be either one, probably depending upon the direction of entry. This would mean that from two molecules of identical length and arrangement those markers entering one cell early could enter another cell very late (on a complementary strand moving in the

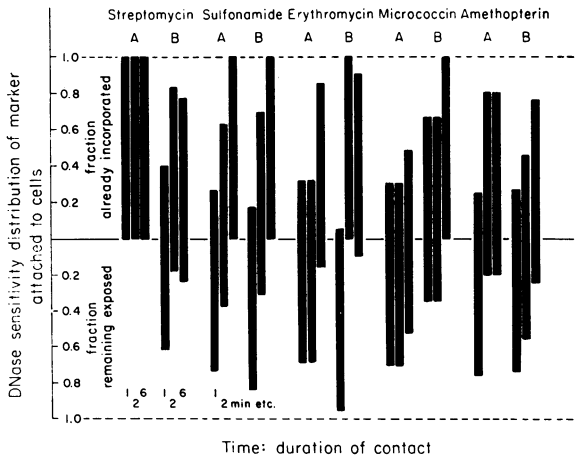


FIG. 4.—Transformants from single-stranded marked DNA fractions A or B, rescued by wild-type DNA. Proportion and rate of achieving DNase insensitivity as in Fig. 3 except that at each time interval, 1, 2, or 6 min, the DNase-insensitive fraction was calculated from swamping dilution (transformants from total attached marker) made at that sime time. Resistance markers: streptomycin (*S* or *str*); sulfonamide (*d* or sul_d[20]); erythromycin (ery_2[28]) micrococcin (*mic*); and amethopterm (*ame*).

opposite direction). The availability in our laboratory of a method of Dr. M. Roger for separating single strands of pneumococcal DNA made it possible to study material in which the entry would presumably always be polarized. These studies revealed that the two strands do not produce equal numbers of transformants; this topic must be presented before taking up their marker distribution.

Intrinsic efficiency of markers in single DNA strands: Fractions of denatured DNA separated from MAK columns[16] did not recover appreciable biological activity unless low-salt (A) and high-salt (B) fractions were mixed before renaturing (Table 1). These separated fractions therefore behave like single DNA strands of opposite polarity; what is most noteworthy is that for all markers tested (eight thus far) this separation is possible. When fractions A and B from a multiply marked DNA are annealed with unfractionated denatured wild-type DNA or opposite fractions from it, they can be partially "rescued" by unmarked DNA strands. As indicated, their biological activity is greatly increased by this rescue. The restoration of 20–30 per cent of native activity in the exceedingly low concentrations (4–10 μg/ml) of DNA fractions used here is reasonable, considering the usual recovery of only 50 per cent of activity in much higher concentrations of unfractionated denatured DNA.

After adequate contact between cells and DNA, the number of transformations is a measure of the rate of recombination (breakage and rejoining minus repair) which is known to be different for different markers and linkage groups.[5] These differences have been attributed to the size span of the marker region[20] or possibly its chemical composition.[20, 28] Ephrussi-Taylor and co-workers[29] found a large number of point mutations in a single genetic region of pneumococcus to fall into two distinct classes of efficiency of genetic incorporation. Lacks confirmed the existence of such classes,[30] finding about four in another genetic region, and proposed that they reflect the different efficiencies of integration of particular nucleotide bases or sequences into the cellular DNA.

As Table 1 shows, there are clear-cut differences between the efficiencies of the same marker allele in the rescued A and B strands. Thus, *ery* activity is low in B strands, and *mic* in A strands; nevertheless, these fractions contribute notably to the renaturation of the opposite fraction. Therefore, it is not likely that the different activities of A and B fractions are simply due to incomplete recovery of marked strands. Each fraction is important for restoration of biological activity. Sec-

TABLE 1

REACTIVATION OF GENETIC MARKER ACTIVITY BY ANNEALING OF SINGLE STRANDS

Strand fraction annealed	Per Cent Native Activity Recovered on Annealing at 65°C					
	str	*sul*	*ery*	*mic*	*ame*	Linkage
A (alone)	2	9	3	2	3	4
B (alone)	2	3	3	11	14	8
A + B	19	21	31	15	22	20
A + wild	9	20	36	5	10	4
B + wild	18	16	7	12	23	14
A + A_{wild}	3	4	3	2	4	1
B + B_{wild}	5	6	7	8	19	3
	Ratio of Activities, Rescued with Wild DNA					
A/B ratio	0.5	1.2	5.1	0.4	0.45	0.3

Annealing carried out with wild unfractionated DNA, or fractions A_{wild}, etc. Activity calculated on basis of marked DNA present; therefore, A + B mixture contains one half the amount of A or B present in the other tests, and should be evaluated against the *average* of A and B separately tested.

ondly, in work to be reported elsewhere, the net activity and the rates of marker expression of native double-stranded DNA appear to reflect a numerical average of the different activities and rates of expression of the separated rescued fractions. For this reason, the *fractions* A and B seem properly to reflect A and B *strands* of native DNA. Accordingly, it seems clear that the efficiency differences are intrinsic, the fractions either giving low incorporation for certain marker alleles or, being incorporated, are more easily "repaired" to the wild-type structure than when they introduce more efficient marker regions. It seems reasonable in line with earlier suggestions[20] and following Lacks[30] to attribute the efficiencies to differences in the base compositions. It should be noted, however, that we are now speaking not of differences between allele efficiencies in double-stranded DNA but of differences in intrinsic efficiencies of the complementary strands, which differ in composition over their whole length as well as at the mutated site or sites.

It is interesting that along the linked marker group, sul_d–*str*–ery_2, the strand showing rapid expression changes over, being A, A, and B, respectively. Presumably, this demonstrates the already suspected transcription of different strands at different regions of the chromosome. Guild and Robison[31] were able to distribute denatured pneumococcal DNA centrifugally in alkaline cesium chloride gradients with sufficient displacement at the extremes to give a 1.5-fold increase of renaturability when fractions were mixed. The rate of expression of one marker appeared considerably faster in the lighter fraction. Szybalski and co-workers[32] have described a fractionation of DNA strands on polyribonucleotides which apparently reflects differences in base distribution.

Since the A and B strands contribute differently to the net activity of native DNA, transformations with both strands present reflect marker abundance and sequences, but only in terms of averages of usually unknown single-strand efficiencies for each allele. Marker-to-marker ratios will no longer serve to normalize for variations in cell incorporation rates for DNA that has been fractionated into different strands or fragments. However, the swamping dilution method outlined above gives us a reference measure of maximal incorporation of a marker against which it is possible to compare rates of cell entry.

Rates of marker entry from single-stranded DNA: Separate cultures can be transformed with the rescued A or B strands; the order of marker entry into the cells should now be fixed by the arrangement of markers and polarity of each strand. (In one of the two possible directions of entry, the marker strand is presumably degraded.) As shown in Figure 4, there are indications that the A and B fractions differ in delivering their markers earlier or later. As time goes on, a greater and greater proportion of the total transforming molecules engaged in entry have delivered their marker in DNase-resistant form, so that the difference between the transformants maximally revealed by terminating reaction with the swamping dilution and those obtained after nuclease termination becomes smaller and smaller. It is seen that different markers require different lengths of time to reach this final rate of entry. Some markers which enter rapidly from the rescued A fraction seem slower to enter from the B fraction, and vice versa. Considering uncertainties about the structure of renatured DNA, including whether it is actually linear,[27] more work will be necessary before gene arrangements can be definitely established, but these new methods seem suitable for determining the distributions of markers

in relation to points at which DNA entry begins. The data obtained thus far and presented in part suggest characteristic distributions for certain markers. The manipulations used in making preparations BT, KP, and C are such that the conditions producing the apparently uniform particles of the chloroform gel C preparations involve very little obvious mechanical shear compared with that which is needed to break the molecules further. As yet, such preparations as BT or KP have not been denatured or fractionated.

Pneumococcal DNA on isolation gently separates into double-stranded particles with narrow distribution about a mean size[33] and showing the evidences presented here that markers have characteristic distributions relative to the ends of the molecules. Pre-existing chain discontinuities which might determine these ends could include single chain interruptions, perhaps separated in complementary strands to give rise to overlapping "cohesive" regions as in λ phage DNA,[34, 35] or connected by interpolated amino acids,[36] cations, or other bases. One of the authors has presented reasons for expecting punctuation by stretches of polydeoxyadenyl nucleotides;[37] easily separated regions might also result from highly methylated stretches. These possibilities in punctuation or breakage would also bear upon the structural basis by which MAK fractionation seems able to recognize differences in the complementary strands.

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