THE DEPENDENCE OF PNEUMOCOCCAL TRANSFORMATION ON THE MOLECULAR WEIGHT OF DEOXYRIBOSE NUCLEIC ACID

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Increasingly quantitative studies of bacterial transformation[1–3] induced by deoxyribose nucleic acid (DNA) have made this a unique tool for analyzing the genetic function of DNA in terms of its molecular structure. By inflicting known amounts of relatively mild damage on transforming DNA and observing the resultant loss in transforming activity, one may gain an intimate knowledge of the molecular features which are responsible for its genetic function. Thus far, it has been chiefly damage induced by various kinds of radiation that has been studied quantitatively,[4–6] but in these cases it has not been possible to assay directly the chemical and physical damage involved. There is, therefore, a need for means by which single types of damage can be imposed and the extent accurately measured, because only in this way can a direct correlation be made between loss in transforming activity and the fractional amount of damage to a particular feature of the molecular structure of DNA.

As a first approach in this direction, we have sought to find the effect on bacterial transformation of breaking the very long DNA molecules into successively smaller pieces. In this case the amount of damage, if confined only to double chain scission, can be obtained at once from the molecular weight of the degraded samples, and the correlation of this with transforming activity should lead to the minimum length of DNA required for the act of transformation if such a minimum length does exist.

Such an investigation requires the availability of samples of transforming DNA covering a range of molecular weight. This requirement has been met by the studies of Doty, McGill, and Rice[7] on the sonic degradation of DNA. They showed that the exposure of solutions of DNA to 9-kilocycle sound waves causes the fragmentation of DNA by the scission of opposite pairs of phosphoester bonds without disturbance of the remainder of the paired polynucleotide strands. As a result, samples of structurally homologous DNA can be prepared which differ physically only in molecular weight.

We report here the preparation of several DNA samples from pneumococcus, the production of sonicates (degraded samples) covering more than a tenfold range in molecular weight, and the correlation of the transforming activity with the molecular weight.

EXPERIMENTAL DETAILS

Bacterial Strains.—The recipient strain used in the transformation experiments was a variant of rough strain R-36A *Diplococcus pneumoniae* derived originally from Type II. In most experiments, the DNA was isolated from a donor strain which carried five unlinked resistance markers: streptomycin, micrococcin, eryth-

romycin, novobiocin, and optochin. The donor strain was obtained by transforming the recipient strain with respect to each of these characters, one at a time. The micrococcin-resistant strain was obtained from Dr. R. D. Hotchkiss, the erythromycin strain (ery_2) from Dr. A. W. Ravin, and the optochin strain from Dr. L. Lerman.

Isolation of DNA.—Active transforming DNA was isolated from mass cultures of the donor strain by lysis of the cells with sodium deoxycholate, followed by alternate deproteinizations with chloroform and precipitations of the DNA with ethanol. Ribonucleic acid was removed by overnight incubation in the cold with ribonuclease, followed by dialysis for 24 hours against 2 *M* NaCl. To insure complete removal of the ribonucleic acid, several precipitations were carried out with isopropanol (0.54 volume fraction) in the presence of 0.3 *M* sodium acetate, according to Simmons. The DNA was never exposed to ionic strengths less than 0.01.

Sonic Degradation.—Sonic degradations were carried out for various lengths of time (1 minute–3 hours) on small volumes (2–3 cc.) of solutions contained in nitrocellulose centrifuge tubes suspended in water within the cup of a Raytheon magnetostriction sonic oscillator (50-watt).

Molecular weights were derived from sedimentation constants ($s_{20,w}^0$) using the relation established by Doty, McGill, and Rice:[7]

$$s_{20,w}^0 = 0.063\, M_w^{0.37}.$$

The sedimentation constants were determined from the median point on the photometer trace of the photographs taken with the ultraviolet absorption optics in the Spinco Model E ultracentrifuge at concentrations less than 30 mg/l. Intrinsic viscosities extrapolated to zero gradient were determined for two undegraded samples and found to be 33 for TP-32-M and 60 for Ca-Str-15 in units of 100 cc/gm. These values led to the same molecular weights as the sedimentation constants when the corresponding intrinsic viscosity–molecular-weight relation[3] was employed. The probable error associated with the molecular weights is ±15 per cent.

In an initial set of experiments, sonic irradiation was carried out with the DNA dissolved in the standard saline-citrate solvent (0.015 *M* NaCl plus 0.015 *M* sodium citrate). The results are shown in Figure 1 as the logarithm of transforming activity plotted against reciprocal molecular weight. Since it had been shown that denaturation temperature of DNA was not lowered by sonic irradiation,[7] we thought at first that indirect effects were absent. However, it was found that the activity of a sample degraded to a given molecular weight was somewhat dependent on the concentration of the DNA solution irradiated with sonic waves. The addition of 5 per cent of *S*-(2-aminoethyl)isothiuronium bromide hydrogen bromide (AET),[8] kindly supplied by Dr. D. G. Doherty, eliminated the concentration dependence and led to samples having higher activity for a given molecular weight. Some results for some samples produced from the same undegraded DNA in the presence of AET are also plotted in Figure 1. As a consequence, 5 per cent AET was used in the preparation of subsequent sonicates. The AET was removed by dialysis after the sonic treatment. The sedimentation constants and derived weight average molecular weights are listed in Table 1.

Transformation.—The samples were assayed for transforming activity, using glycerol-treated recipient cells stored at −15° C.[2] Exposure of the recipient cells

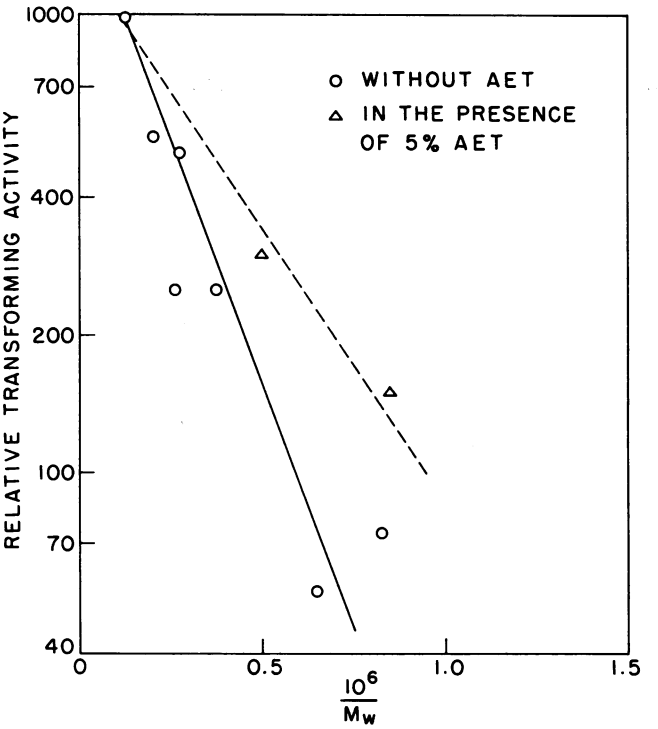

FIG. 1.—Logarithm of the relative streptomycin-transforming activity of sonic fragments prepared in the absence and presence of AET plotted against the reciprocal molecular weight.

TABLE 1

SEDIMENTATION CONSTANTS, MOLECULAR WEIGHTS, AND RELATIVE TRANSFORMING ACTIVITIES OF UNDERGRADED AND SONICATED SAMPLES OF DNA

Sample	$(s_{20,w})^0$ Median	$M_w \times 10^{-6}$	Relative Activity
TP-34-M:			
Control	23	8.0	1,000
Sonicate	9.3	0.87	100
Ca-Str-15:			
Control	23	8.0	400
Sonicate (1)	12.7	2.1	112
Sonicate (2)	10.7	1.2	72
TP-32-M:			
Control	15.5	3.5	150
Sonicate (1)	14.8	2.8	132
Sonicate (2)	13.1	2.0	102
Sonicate (3)	10.3	1.1	41
Sonicate (4)	8.6	0.72	13
Sonicate (5)	6.3	0.32	0.3

to DNA at 32° C. was terminated after 30 minutes by the addition of deoxyribonuclease (Worthington, crystalline). The cells were then incubated at 37.5° C. for 90 minutes and scored in appropriate selective media. The DNA concentration used in transformation was always sufficiently low to insure that the number of transformants was proportional to the concentration.[5]

Comparisons of the activity of the sonicates with the undegraded samples were made by measuring the relative number of transformants induced in aliquots of a single recipient culture by equal weight concentrations of the various DNA samples. In nearly all cases the conditions for assay were adjusted so that at least 100 transformed cells would be counted. As a consequence, the limits of error in the assays can be set as ±20 per cent from the 95 per cent confidence limit of the Poisson distribution. The maximum efficiency of transformation, using undegraded samples, was about 1 per cent.

RESULTS

Undegraded Samples.—A number of different samples of DNA have been made from pneumococci. The molecular weights fell within the range of 3–8 million, and the activities (streptomycin transformation), with occasional exceptions, spanned a range of 10. There was a rough correlation between activity and molecular weight, but occasionally a sample of unusually low activity was prepared. Two samples used for the preparation of sonicates had the highest activity and the highest molecular weight of all the samples prepared (Ca-Str-15 and TP-34-M). It is interesting to note that the molecular weight in these cases matched the highest values of molecular weight obtained from calf thymus and other sources.[9, 10] The other sample used (TP-32-M) was typical of the majority of preparations we have made. The highest streptomycin-transforming activity was shown by sample TP-34-M, and it has been assigned a value of 1,000. All other activities are recorded relative to this value.

Sonicates.—From the three original DNA samples just mentioned, a total of 8 sonicates was prepared, characterized, and assayed. The logarithm of the relative activity in streptomycin transformation of these 11 samples is plotted in Figure 2 as a function of reciprocal molecular weight. Since substantial evidence has been obtained[11, 12] to show that the molecular-weight distribution of the sonicates is essentially that of the most probable distribution (see below), it follows that the reciprocal molecular weight is directly proportional to the fraction of bonds broken. Consequently, this quantity also serves as an abscissa and is indicated in Figure 2. Simultaneous assays were carried out on most of the samples for transformation to erythromycin and micrococcin resistance. The relative activities were the same within probable error. These results demonstrate, therefore, that, although the undegraded samples differ among themselves, the sonicates of any undegraded sample show the same decay in activity with decreasing molecular weight for three different genetic markers. This result—a direct proportionality between the logarithm of activity and the fraction of bonds broken—is the simplest possible, but its interpretation depends upon an analysis of the transformation process.

Analysis of First-Order Decay as a Function of Reciprocal Molecular Weight.—An essential feature of the transformation assays in this work was the use of recipient cells in such a state that transformation in them is induced at a constant rate over the period of exposure to the DNA (30 minutes). Fox and Hotchkiss[2] have pointed out that, under these conditions, the rate of induction of transformation shows a dependence on DNA concentration resembling that of the Michaelis-Menten mechanism of enzyme kinetics. Thus they suggest that a rapidly established equilibrium is set up between free DNA and DNA reversibly bound at a

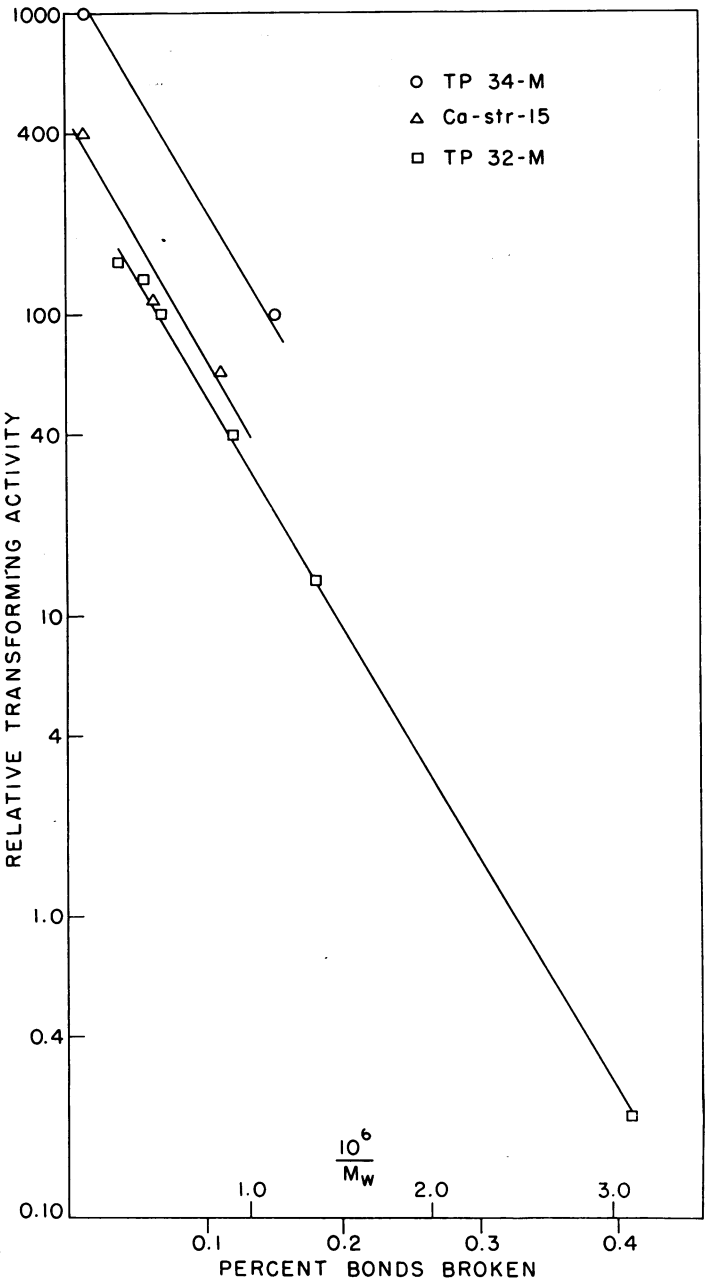

FIG. 2.—Logarithm of the relative streptomycin-transforming activity of sonic fragments prepared in the presence of AET plotted against the reciprocal molecular weight and per cent bonds broken.

bacterial site. Transformation is then proportional to the irreversible incorporation of DNA from the DNA-site complex:

$$\text{DNA} + \text{site} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{DNA-site complex} \xrightarrow{k_3} \text{incorporated DNA} + \text{site}.$$

It is convenient to discuss our results in terms of this mechanism, but it must be kept in mind that the concentration of DNA must now be considered on a molar basis rather than on a weight basis. The latter choice is satisfactory for conventional experiments where the ratio of the weight and molar concentrations is a constant, but it does not hold when the molecular weight is varied, as in our case.

As in enzyme kinetics it is assumed that the concentration of DNA, the analogue of the substrate, is much greater than the concentration of bacterial sites. In this case the velocity of incorporation is given by

$$v_i = \frac{k_3 \text{ site [DNA]}}{(k_2/k_1) + \text{[DNA]}}, \tag{1}$$

where DNA represents the *molar* concentration of DNA. If we now assume that (1) the same values of the three rate constants apply to all molecular species of DNA, (2) a fraction z of the DNA molecules contains the active markers, and (3) transformation will occur with a probability p when an active marker enters the cell, the rate of transformation, v_7, is given by

$$v_t = pzv_i = \frac{pzk_3 \text{ site [DNA]}}{(k_2/k_1) + \text{[DNA]}}. \tag{2}$$

When the concentration of DNA is sufficiently small, $[\text{DNA}] \ll k_2/k_1$, and

$$v_t = pz \frac{k_3k_1}{k_2} \text{[site] [DNA]}. \tag{3}$$

This region of low DNA concentration corresponds to the linearly increasing portion of the transformation versus concentration plot, where the assays reported here were performed. When the activities of two samples of DNA are determined, using aliquots of the same culture of recipient cells, it is the ratio of values of the product, $pz(k_3k_1/k_2)$ [DNA], which is obtained. The observed variation of transforming activity with molecular weight may therefore result from a first-order decay in one or more than one component of this product. If, for example, the sonically induced chain scission had only the effect of destroying active markers when a scission occurred within them, the effect would be described as a first-order decay in z[DNA], since this product equals the number concentration of markers. Alternatively, if there were a minimum molecular size required to form the DNA-site complex, the molar concentration of DNA would have to be corrected for the amount that did not have the minimum molecular weight. That is, [DNA] would mean the molar concentration of material having molecular weights in excess of the minimum value.

We consider these two possibilities—decay in number of active markers and gradual conversion of DNA into pieces too small to form the DNA-site complex—to be the most likely cause of the observed fall in activity with the fraction of bonds broken in the sonicates. Analyses of these two effects lead to the conclusion that both will display a first-order, logarithmic, decay as a function of the fraction of bonds broken. We summarize here the argument for the case of inactivation due to damage of markers and postpone until later the case involving a minimum length for attachment.

Since the inactivation of a marker is assumed to take place when a double-chain

scission occurs within it, this event may be considered a hit and the conventional target theory applied. In this context, let H be the number of hits per gram of DNA, N the number of markers per gram of DNA, S the number of double-chain scissions per gram of DNA, and M_c the molecular weight of the sequence that constitutes the marker. A fraction $1/e$, or 0.37, of the markers will survive when the number of hits becomes equal to the number of markers. Using the subscript 37 to denote quantities relating to the 37 per cent dose, $H_{37} = N$. For the 37 per cent dose, the fraction of double-chain scissions which occur within active sequences is NM_c; the total number of breaks which occur within active sequences is therefore given by $H_{37} = S_{37}\, NM_c$. Equating these two expressions for H_{37} gives $M_c = 1/S_{37}$.

We have reason to believe that all our DNA samples, both degraded and undegraded, have molecular-weight distributions which may be represented approximately by a "most-probable" or Flory distribution.[13] For this distribution, q, the fraction of bonds broken, is given by

$$q = 2/x_w = 2M_0/M_w,$$

where x_w is the weight average degree of polymerization, M_w is the weight average molecular weight, and M_0 is the molecular weight of a single nucleotide pair. Since S is given by

$$S = qN_0/M_0 = 2N_0/M_w,$$

where N_0 is Avogadro's number, a plot of the logarithm of relative activity versus the reciprocal weight average molecular weight should be a straight line, as is indeed observed. For the 37 per cent dose,

$$S_{37} = q_{37}N_0/M_0;$$

therefore,

$$\begin{aligned} M_c &= M_0/q_{37}N_0 \text{ (in grams)} \\ &= M_0/q_{37} \text{ (in molecular-weight units).} \end{aligned}$$

If it is recognized that our starting material was not infinitely long DNA, but had a finite fraction of bonds already broken, this may be denoted by q_{100}. This is then used to correct the critical molecular weight:

$$M_c = M_0/(q_{37} - q_{100}) = 660/(q_{37} - q_{100}).$$

Using this result and the slope of the lines in Figure 2, one arrives at the value of 1.0 million for M_c.

The alternative analysis, involving the conversion of DNA to pieces too small to form the complex, leads to a result that is formally the same as this one, provided that the molecular-weight distribution remains the most probable one. Consequently, if it is considered that the loss in activity due to sonication occurs only as a result of reduction in molecular size below that required for attachment, one is led to expect that the logarithm of the relative activity will fall linearly with the reciprocal molecular weight and that the observed slope corresponds to a critical molecular weight of 1 million for attachment.

DISCUSSION

Thus two alternative interpretations of the data are possible. The critical value of the molecular weight determined is the same in each case, but it has two quite different meanings. The fact that it is the same for three different markers suggests that it represents a common requirement for transformation that is independent of the markers themselves, which, a priori, would be expected to be of smaller and variable molecular weights. This argues that the critical size refers to the attachment size. On the other hand, the active marker may be large enough to have been significantly damaged by the chain scissions. A discussion of this possibility requires a clarification of the term active marker used above. Our use of this term in connection with equation (2) implies that this is the minimum length of DNA required for transformation after passage through the attachment site. Its minimum size would presumably refer to the minimum length of DNA required to contain the nucleotides that are altered in the mutation that gives rise to the altered strain. From the work of Benzer it appears that this *may* be as small as a few nucleotides. However, this minimum size, which may be called a mutational site, could be considerably smaller than the active site. For example it may have to be located well away from the ends of a DNA molecule in order to participate in recombination or the act of recombination itself may have a minimum size requirement. Thus although the mutational site or genetic marker itself may well be so small that it escapes damage during the sonication, the region required for its expression after incorporation may be large enough to contribute to the critical size measured in these experiments. The simultaneous operation of both of these effects would still lead to the observed behavior. We are therefore, at present, undertaking experiments designed to resolve the relative contribution of each of these processes to the observed decay in activity.

It follows from these considerations that the critical molecular weight of 1.0 million obtained here refers to the minimum molecular weight for attachment or for incorporation into the genetic material of the cell or to the combined effect of these two limiting processes. It is clear, therefore, that the critical molecular weight found here does not bear any direct relation to the radiation sensitive volume obtained from radiation inactivation experiments[4–6] and should not be compared directly with these other results.

SUMMARY

Exposure of solutions of transforming factor to 9-kilocycle sound waves causes scissions of the molecules with a concomitant fall in transforming activity. The addition of *S*-(2-aminoethyl)isothiuronium bromide hydrogen bromide during sonic treatment eliminated inactivation of transforming factor by secondary effects. By plotting the logarithm of the relative transforming activity of the sonic fragments versus the reciprocal molecular weight and analyzing the curves by the conventional target theory, the critical molecular weight for streptomycin, erythromycin, and micrococcin was found to be 1.0 million. The value may reflect either the minimum size required for effective attachment to a bacterial site or a combination of this and the minimum size required for genetic incorporation following entrance into the bacterial cell.

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[13] This distribution is the one that occurs when an infinitely long chain is degraded by random scisson. The weight fraction of molecules having a degree of polymerization x is

$$W_x = xq^2(1 - q)^{x-1},$$

where q is the probability of a bond having been broken.

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*OBSERVATIONS IN MYELIN STRUCTURE: INCISURES AND NODAL REGIONS**

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Biophysical studies employing polarization optics, X-ray diffraction, and electron microscopy have provided valuable information concerning the layered structure of nerve myelin at the molecular level.[1–4] Electron microscope studies of the myelination process by Geren[5] led to the concept that the lipid-protein layered structure, which repeats very regularly in the radial direction, is produced by the wrapping about the axon of many folds of the surface membrane of the Schwann cells, which come to surround the outgrowing axon. Geren's findings have been confirmed by a number of investigators (see particularly Robertson[6]), so that the general concept has now been well established. However, the structure of the myelin at the Schmidt-Lanterman incisures and at the nodes of Ranvier remains to be determined, and it is with this problem that the author's investigation deals. A