Reprinted from *J. Mol. Biol.* (1968) **37**, 87–98.

Inactivation of *Haemophilus influenzae* Transforming DNA by Spleen Acid Deoxyribonuclease

Giorgio Bernardi and Marie-Louise Bach

APPENDIX

Estimation of the Ratio of Total Bonds broken to Bonds broken by Diplotomic Degradation in Native DNA digested by Spleen Acid DNase

J. Mol. Biol. (1968) **37**, 87–98

Inactivation of *Haemophilus influenzae* Transforming DNA by Spleen Acid Deoxyribonuclease†

GIORGIO BERNARDI AND MARIE-LOUISE BACH

Centre de Recherches sur les Macromolécules
Strasbourg, France

(*Received 16 February 1968, and in revised form 1 July 1968*)

The inactivation of transforming DNA from *Haemophilus influenzae* by spleen acid DNase has been investigated. At comparable levels of bond breakage, this enzyme is much more effective as an inactivator than pancreatic DNase, *Escherichia coli* DNase or sonication. For example, in order to obtain a Poisson average of one inactivating event per cathomycin marker (37% survival), more than fifty breaks per DNA molecule of 12×10^6 daltons are needed in the case of pancreatic DNase degradation, whereas less than two to four breaks by acid DNase produce the same effect. This implies that the spleen enzyme can inactivate a marker in the absence of a direct lesion.

Competition experiments have shown that this peculiar inactivation is not due to impaired uptake of partially degraded DNA by the bacterial cells. These experiments have provided, in addition, evidence that uptake does not depend upon the nature of the ends (3′ or 5′ phosphates) and is not hindered by the presence of "hidden breaks" or "dangling ends", but only depends upon the size of DNA.

Degradation by acid DNase hinders, therefore, the integration of DNA into the host cell genome. This phenomenon is probably due to the 3′ phosphate ends formed by acid DNase, since *E. coli* DNase and sonication, which also mainly degrade native DNA by a diplotomic mechanism, like acid DNase, but form 5′ phosphate ends, are much less effective as inactivators at comparable levels of degradation.

1. Introduction

Investigations on the inactivation of transforming DNA by enzymic, chemical and physical agents are extremely useful for our understanding of both the fundamental steps of bacterial transformation: DNA uptake by the cell, and DNA integration into the host genome. Enzymic inactivation of DNA is due to a single cause, namely, phosphodiester bond breakage; this is an important advantage over inactivations caused by physical and chemical agents, since these depend upon multiple and generally ill-known reactions.

The enzymic inactivation of transforming DNA has been investigated so far only in the case of pancreatic DNase‡ (Zamenhof, Alexander & Leidy, 1953; Lerman & Tolmach, 1957; Goodgal, 1961; Stuy, 1961; Bodmer, 1966), an enzyme degrading

† This is paper VII in the series "Studies on acid deoxyribonuclease"; paper VI is Cordonnier & Bernardi (1968).

‡ Abbreviations used: DNase, deoxyribonuclease; S, streptomycin marker; Ca, cathomycin marker.

DNA according to a haplotomic mechanism† (Thomas, 1956; Schumaker, Richards & Schachman, 1956) and forming oligonucleotides having a 5′ phosphate end (Sinsheimer & Koerner, 1951).

In the present work, we have investigated the inactivation of *Haemophilus influenzae* DNA by spleen acid DNase. This enzyme has been extensively studied in our laboratory during the past few years and our work has been recently reviewed (Bernardi, 1968*a*,*b*). Spleen acid DNase splits native DNA according to both diplotomic and haplotomic mechanisms (Bernardi & Sadron, 1961; 1964*a*,*b*; Young & Sinsheimer, 1965), forming oligonucleotides carrying a 3′ phosphate end (Koerner & Sinsheimer, 1957).

We thought that the inactivation of transforming DNA by the spleen enzyme might be different from that known for pancreatic DNase because of the different mechanisms of action and specificities shown by the two enzymes with respect to the position of the terminal phosphates.

In fact, we have not found any feature in the inactivation of transforming DNA which may be specifically related to its diplotomic mechanism of action; this is not surprising in view of the fact that transformation occurs by integration of single-stranded and not of double-stranded DNA in the host genome. Instead, we have observed a very interesting phenomenon of long-range inactivation, which seems to be due to the presence of 3′ phosphate ends in the DNA partially degraded by acid DNase.

2. Materials and Methods

(a) *Cells and transformation*

Haemophilus influenzae (Rd) cells were used throughout. Resistances to streptomycin (25 μg/ml.) and cathomycin (1 μg/ml.) were used as genetic markers; these were chosen since they are linked markers.

The transforming assay used in this work has been described elsewhere (Bach, Luzzati & Chevallier, 1966). In a few instances, assays were carried out according to Stuy (1961), allowing the phenotypic expression to take place in solid rather than in liquid media; similar results were obtained in both cases, in agreement with a previous report by Goodgal (1961). The transformation experiments were performed at a saturating DNA concentration (1 μg/ml).

Competition experiments were done by measuring the transforming activity of a DNA sample (E 465) carrying the erythromycin marker in the presence of DNase-treated DNA samples (N 2/A 366; see below) carrying the cathomycin marker. The competitive activity of the intact cathomycin DNA sample was taken as 100%. All competition experiments were done at two different concentrations of cathomycin DNA.

(b) *DNA*

DNA preparation was carried out as described by Bach *et al.* (1966). The properties of the two DNA samples which were submitted to DNase degradation are given in Table 1.

(c) *Enzymes*

Pancreatic DNase was once crystallized Worthington DNase (lot 932). The enzyme was dissolved in 0·1 M-phosphate buffer, pH 6·8, 0·02 M-$MgCl_2$ (buffer A) at a concentration of about 0·5 mg/ml. The stock solution was diluted 100 times with the same buffer and 10 μl. of this dilution were added to 30 ml. of DNA solution; therefore, the final enzyme concentration was about 1·5 mμg/ml.

† We use the terms diplotomic and haplotomic to describe the mechanisms of degradation of double-stranded DNA involving, respectively, the simultaneous scission of both strands at the same level or the scission of only one strand at a time (Bernardi, 1968*a*,*b*). The first mechanism leads to "single-hit" kinetics, the second one to "double-hit" kinetics.

TABLE 1

Properties of DNA samples

DNA sample	T_m	Hyper-chromicity at 100°C	s†	ml./g‡	M_s§ ($\times 10^{-6}$)	$M_{[\eta]}$§ ($\times 10^{-6}$)	M_{LS}‖ ($\times 10^{-6}$)	Markers
SKN 564	87°8	40%	25·0	6030	12	11·5	8·3	Strepto-mycin Kanamycin Cathomycin
N2/A 366	88°	—	26·0	6610	13·2	13·0	10·0	Cathomycin

† As determined at a concentration of 2·5 mg/100 ml. in a Spinco model E instrument equipped with ultraviolet optics.

‡ As determined after extrapolation to zero velocity gradient and zero concentration. Solvents were A and B for sample SKN 564 (which in solvent C showed a value of 6330 ml./g), B and D for sample N2/A 366 (see Materials and Methods for composition of solvents).

§ Molecular weights calculated from s and $[\eta]$ values, respectively, using the relationships of Eigner & Doty (1965).

‖ Weight-average molecular weight as determined by light-scattering. It is known (Froelich, Strazielle, Bernardi & Benoit, 1963) that light-scattering molecular weights are under-estimated for DNA samples having molecular weights higher than 6×10^6.

Acid DNase was prepared from hog spleen according to the method of Bernardi & Griffé (1964), as modified by Bernardi, Bernardi & Chersi (1966). Preparations HS 10, 13, 16 and 17–18 were used. The enzyme stock solutions (1·5 mg/ml. in 0·1 M-acetate buffer, pH 5·0) were diluted 10,000 times with 0·15 M-acetate buffer–0·01 M-EDTA, pH 5·0 (buffer B) containing 0·05% beef heart cytochrome *c* (Sigma, type V); 50 μl. of the diluted solution were added to 30 ml. of DNA solution; therefore, the final concentration was about 0·15 mμg/ml. When DNA solutions in 0·01 M-phosphate buffer, pH 6·7 (buffer C) were used, the final enzyme concentration was 35 times higher.

E. coli DNase (endonuclease I of Lehman, Roussos & Pratt (1962)) was prepared according to Cordonnier & Bernardi (1966; and manuscript in preparation). The enzyme stock solution was diluted with 0·066 M-Tris buffer (pH 7·6)–0·006 M-$MgCl_2$ (buffer D).

(d) *DNase degradation*

This was followed by viscometry using a four-bulb viscosimeter built according to Eigner (1960). The solvents used are mentioned above. DNA concentration was equal to 50 μg/ml. Except where otherwise stated, the correction for the concentration dependence was applied, and the temperature used was 28° ± 0·01°C.

As in previous work (Bernardi & Cordonnier, 1965), only one bulb of the viscosimeter was used in routine experiments; in the present investigation, the bulb used was associated with an average shear gradient of about 75 sec^{-1}; the dependence of DNA viscosity on shear was determined for every solvent used, at different stages of enzymic degradation. This allowed us to correct the viscosities determined at a finite shear gradient. Since only the initial viscosity decrease was studied in this work, the corrections used for the initial and the digested samples did not differ very much from each other. The correction factors were 1·21 and 1·19 for buffer A, 1·21 and 1·15 for buffer B and 1·19 and 1·13 for buffer C, the first figure being the correction factor for the initial, and the second for the final sample used in the transformation assays (see below), respectively. The average velocity gradients associated with each bulb were calculated using the equation of Kroepelin (1929); values ranging from 90 to 20 sec^{-1} were found.

The standard experimental procedure was the following. Enzyme was added to 30 ml. of DNA solution, which had been clarified as described by Bernardi (1964; except that no shaking with chloroform–isoamyl alcohol was used), and kept in a flask immersed in the thermostatically controlled bath of the viscosimeter. A 20-ml. portion of the digestion

mixture was immediately introduced into the viscosimeter and measurements were started. At regular time-intervals 1- or 5-ml. portions were withdrawn from the flask and pipetted into test tubes containing 1 ml. of chloroform–isoamyl alcohol (5:1, v/v; Sevag, 1934) and 1 or 5 ml. M-NaCl when DNA was in buffer A, C or D; M-potassium phosphate buffer (pH 6·8), replaced M-NaCl when DNA was in buffer B. Tubes were shaken by hand for 1 min to inactivate the enzyme and stored at 0°C. These DNA samples were used for the transformation experiments.

(e) *Molecular weights and kinetic data*

Weight-average molecular weights were calculated from the intrinsic viscosities using the relationship of Eigner & Doty (1965):

$$[\eta] = 6{\cdot}9 \times 10^{-4}\, M^{0{\cdot}70} \qquad (1)$$

which is valid in the region 2 million to 130 million daltons†.

The viscosity results obtained during the enzymic degradations were treated, as in previous work (Bernardi & Sadron, 1964*a*,*b*; Bernardi & Cordonnier, 1965) according to Schumaker *et al.* (1956). The apparent number of strands, n, was calculated from the following equation (Cavalieri & Rosenberg, 1961):

$$\log \frac{1-R}{R} = n \log t + \text{constant} \qquad (2)$$

In this formula, R is equal to M_t/M_0 or to η_t/η_0; M_t, η_t, M_0, η_0 are the weight-average molecular weights (calculated according to equation (1), or the reduced viscosities (at a shear gradient of about 75 sec^{-1}) at times t and 0 of digestion, respectively.

The number of scissions was calculated according to Charlesby's (1954) formula (see footnote to Table 1 A of the Appendix) from the weight-average mol. wt.

3. Results and Discussion

(a) *Kinetics of degradation*

The kinetics of enzymic degradation were investigated by following the decrease in viscosity of *H. influenzae* DNA produced by acid DNase at pH 5·0 and 6·7, and by pancreatic DNase. Two different experimental conditions were used in the digestions with the spleen enzyme, because its pH optimum is close to 5·0 at $\mu = 0{\cdot}15$, but close to 7·0 at $\mu = 0{\cdot}01$ (Shack, 1957; Bernardi & Sadron, 1964*b*). In the latter conditions the enzymic activity is much lower than in the former. It might be expected that the mechanism of degradation of the enzyme is different under the two different conditions.

The results obtained are shown in Figure 1, where data are plotted as M_0/M_t *versus* digestion time. The same data have been re-plotted in the form $\log\left(\frac{1-R}{R}\right)$ *versus* $\log t$ in Figure 2, and values for the apparent number of strands have been calculated from the slopes of the straight lines obtained. The kinetic results show that *H. influenzae* DNA is initially degraded by acid DNase according to a diplotomic mechanism, whereas data obtained with pancreatic DNase show the typical time lag and a haplotomic mechanism of degradation.

These results need not be discussed in detail, since the data obtained with pancreatic DNase are in agreement with the accepted mechanism of degradation (Thomas, 1956; Schumaker *et al.*, 1956), involving random breaks on one or the other of the

† We did not use the relationship obtained by Richards & Bernardi (manuscript in preparation; see also Bernardi & Sadron, 1964*a*), which was obtained by determining molecular weights by intrinsic viscosity at zero shear gradient and light scattering for two series of calf thymus DNA preparations degraded by acid DNase, since this was established over the lower 0·4 to 4×10^6 molecular weight range.

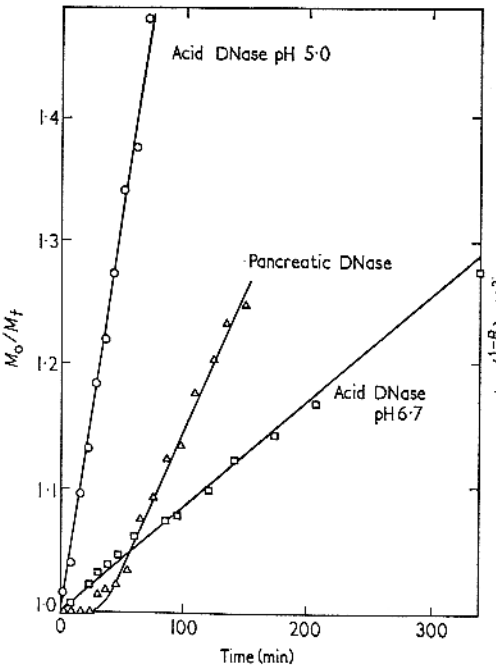

FIG. 1.

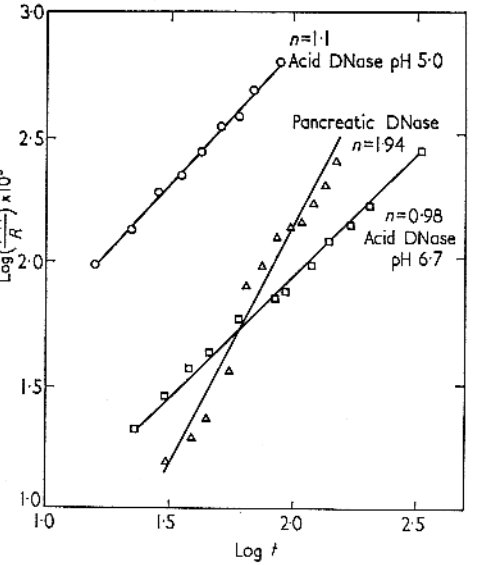

FIG. 2.

FIG. 1. Digestion of *H. influenzae* DNA (SKN 564) by acid DNase at pH 5·0 (○) or 6·7 (□), and by pancreatic DNase (△). M_0/M_t data derived from viscosity measurements are plotted against digestion time.

FIG. 2. Digestion of *H. influenzae* DNA (SKN 564) by acid DNase at pH 5·0 (○) or 6·7 (□), and by pancreatic DNase (△). Data of Fig. 1 are plotted according to Schumaker *et al.* (1956). The values of the slopes giving *n*, the apparent number of strands, are marked next to each straight line.

two DNA strands; and the experiments performed with acid DNase, both at pH 5·0 and 6·7, confirm the existence of the diplotomic mechanism of action of the enzyme seen during the initial degradation of native DNA's from other sources (Bernardi & Sadron, 1961; 1964*a*,*b*; Young & Sinsheimer, 1965).

(b) *Inactivation of transforming DNA*

Figure 3 shows the drop of specific viscosity and the loss of transforming activity occurring when *H. influenzae* DNA is digested by the two DNases. In order to make the comparison easier, the time scales were chosen in such a way as to get comparable viscosities at about the same distances along the abscissae. In all cases, the cathomycin marker was inactivated more slowly than the streptomycin marker; this finding is in agreement with similar results by Goodgal (1961) and Stuy (1961). A different presentation of the results of Figure 3 is given in Figure 4, where transforming activity is plotted against molecular weight. For the sake of simplicity, only the cathomycin data are shown.

There is a striking difference in the inactivation of the genetic markers by acid DNase and pancreatic DNase. In fact, at a survival level of the cathomycin marker equal to 37%, that is, when a Poisson average of one inactivating event has taken place in this marker†, no molecular scission has yet occurred in the degradation by

† The term "marker" indicates here the "marker target".

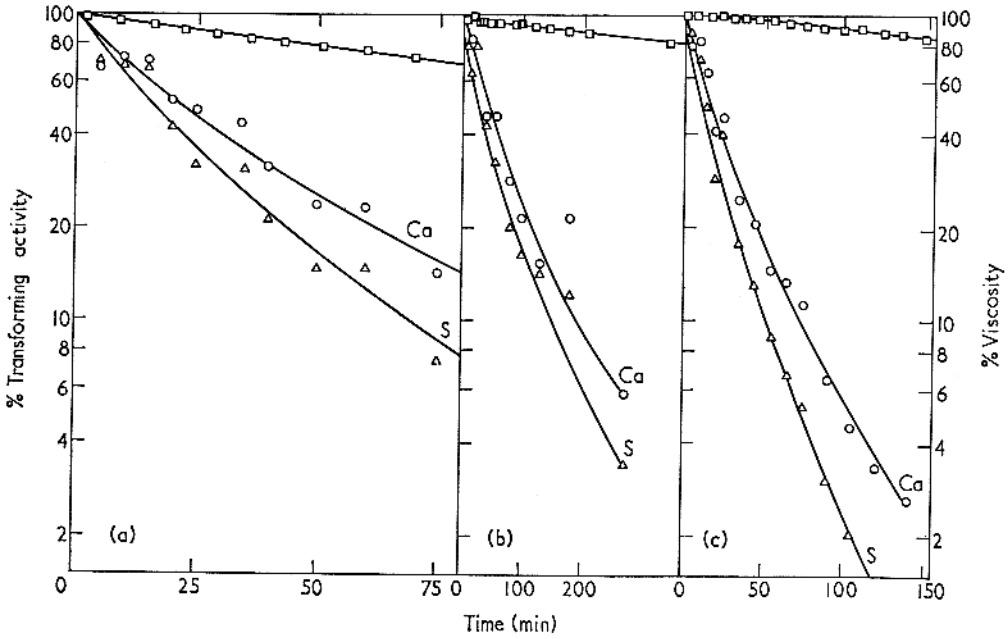

FIG. 3. Viscosity drop (□) and inactivation of the cathomycin (Ca; ○) and streptomycin marker (S; △) during the degradation of *H. influenzae* DNA (SKN 564) by acid DNase at pH 5 (a) or 6·7 (b), and by pancreatic DNase (c).

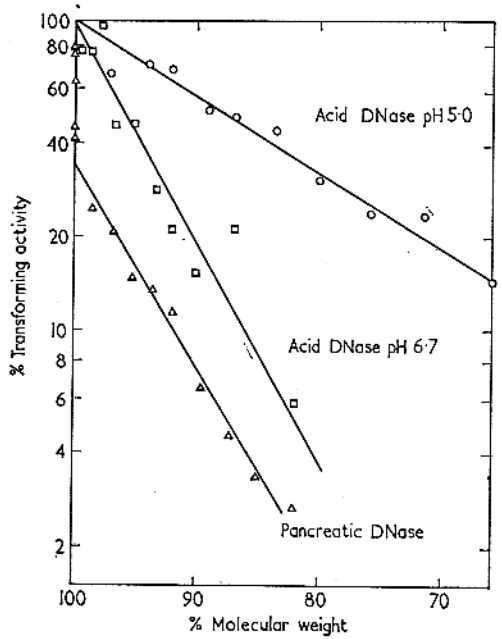

FIG. 4. Inactivation of the cathomycin marker plotted against DNA molecular weight. Data were obtained during the degradation of *H. influenzae* DNA (SKN 564) by acid DNase at pH 5 (○) or 6·7 (□), and by pancreatic DNase (△).

pancreatic DNase (in agreement with the early observations of Zamenhof *et al.*, 1953).

In contrast, at the same inactivation level, 0·63 and 0·22 scission per weight average DNA molecule has occurred in the digestions by acid DNase at pH 5·0 and 6·7, respectively. These values refer to the results presented in Figure 4; by averaging all our results, 0·55 and 0·30 scission per molecule were obtained at the two pH values, respectively. These findings provide independent evidence that acid DNase degrades DNA according to both diplotomic and haplotomic mechanisms, under both experimental conditions. In fact, one inactivating event takes place in a given marker at a degradation level at which DNA molecules have undergone an average of less than one scission, a result which implies that "hidden" breaks are also caused by the enzyme. The results obtained also indicate that, if the same total number of breaks is required to produce the same extent of inactivation, more haplotomic breaks occur at pH 6·7 than at pH 5·0.

(c) *Inactivation of the linked streptomycin and cathomycin markers*

Figure 5 shows the inactivation caused by acid DNase at pH 5·0 and 6·7 and by pancreatic DNase, respectively, as determined on the streptomycin and cathomycin markers together; the inactivation curves are compared with those obtained by

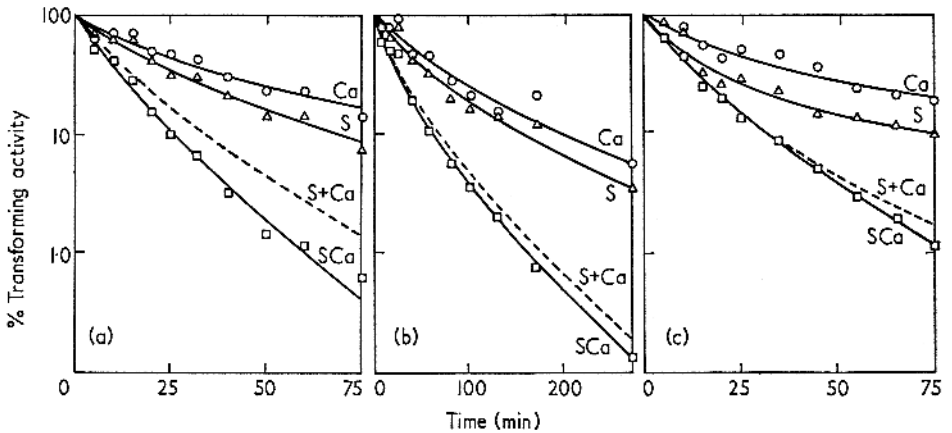

FIG. 5. Inactivation of the cathomycin marker (Ca; ○), the streptomycin marker (S; △), and the two linked markers together (SCa; □), during the degradation of *H. influenzae* DNA (SKN 564) by acid DNase at pH 5 (a) and at pH 6·7 (b), and by pancreatic DNase (c). The broken lines (S + Ca) indicate the sums of the inactivations of the markers tested above.

adding the inactivation measured separately for the two markers. Our failure to find any significant unlinking in pancreatic DNase-treated DNA is in agreement with similar results of Goodgal (1961), but contradicts the findings of Stuy (1961). Some extent of the unlinking of markers is found in the acid DNase digest obtained at pH 5·0. Interestingly enough, unlinking is almost non-existent when spleen DNase degradation is done at pH 6·7, under conditions where the relative number of diplotomic breaks seems to be lower.

(d) *Relationship between inactivation and number of breaks*

The basic results of the present work are those shown in Figure 4. They allow one to estimate the number of breaks which is required in order to obtain a given level of inactivation.

As just mentioned, in the case of pancreatic DNase degradation, the level of one hit per marker is reached near the end of the lag time. At this point not less than 50 phosphodiester bond breaks have occurred per molecule of $M_w = 12 \times 10^6$, a value estimated from unpublished data of Schumaker *et al.* (1956). This number is large enough to account for our finding of one hit per cathomycin marker, the size of which would be estimated as equal to about 3×10^5 daltons.

The situation is completely different in the case of the degradation of DNA by acid DNase at pH 5·0. Here the ratio r of *total* bonds broken to bonds broken according to the diplotomic mechanism can be estimated as lying between 1·5 and 3 (see Appendix). The *total* number of bonds broken by acid DNase at pH 5·0 at a degradation level corresponding to one inactivation event per cathomycin marker therefore lies below 2 to 4 per molecule of $M_w = 12 \times 10^6$. This value is too low to account for one hit per cathomycin marker according to the target theory, and necessarily leads to the surprising conclusion that inactivation must arise, to a very large extent at least, from a phenomenon which does not require a direct lesion of the marker.

(e) *Mechanism of inactivation by acid DNase*

The indirect inactivation by acid DNase must occur at one or both of the two basic steps in transformation: DNA uptake and DNA integration.

(i) The first possibility is that degradation by acid DNase, in contrast to that by pancreatic DNase, impairs the uptake of DNA by the cell.

Competition experiments were performed to check this hypothesis. It is well known that competition takes place at the uptake and not at the integration level (Schaeffer, 1957) and therefore may be used for this purpose. The competitive activity of transforming DNA during the degradation by acid DNase (at pH 5·0) and by pancreatic DNase was investigated and the results are shown in Figure 6. They indicate that, at comparable molecular weights, the competitive activities of DNA samples digested by the two enzymes are the same, thus ruling out the possibility that DNA digested by acid DNase cannot be so readily taken up by the cell. Thus, uptake does not depend upon the nature of the ends (3′ or 5′ phosphate), and is not impaired by the presence of "hidden breaks" or "dangling ends", which exist in DNA partially degraded by pancreatic DNase. Sonicated DNA (which has 5′ phosphate ends, see below) of molecular weight close to $1{\cdot}5 \times 10^6$ has a competitive activity which is very much the same as that shown by enzymically degraded DNA of comparable molecular weight (Bach, 1968). In agreement with Lerman & Tolmach (1957), it was found that the decrease in transforming activity for a given extent of DNA degradation is much greater (by a factor of about five) than that of DNA uptake.

(ii) The second possibility is that integration of the transforming DNA fragments produced by acid DNase is hindered. This may be thought to be either somehow related to the diplotomic mechanism of action of the spleen enzyme or to depend upon the presence of 3′ phosphate ends in the fragments. In order to distinguish between these two possibilities, the inactivation of transforming DNA by acid DNase was compared with those caused by *E. coli* DNase and by sonication.

Degradation of DNA by E. coli *DNase* was studied, since this enzyme degrades native DNA mainly according to a diplotomic mechanism, like the spleen enzyme (Bernardi & Cordonnier, 1965; Studier, 1965), but forms oligonucleotides terminated in 5′ phosphates, like pancreatic DNase (Lehman *et al.*, 1962). The results shown in Figure 7 indicate that, at comparable levels of degradation, *E. coli* DNase is about

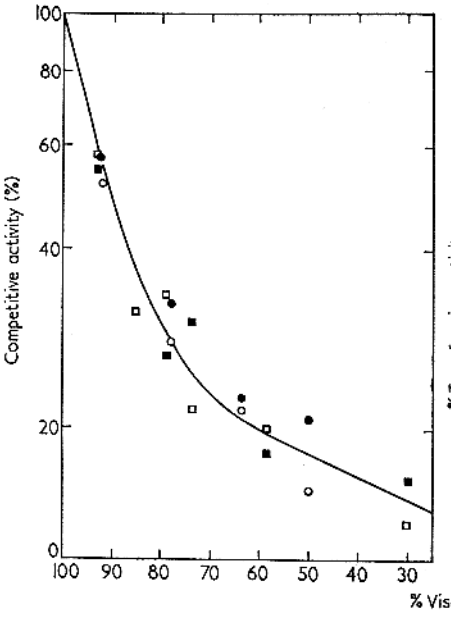

FIG. 6.

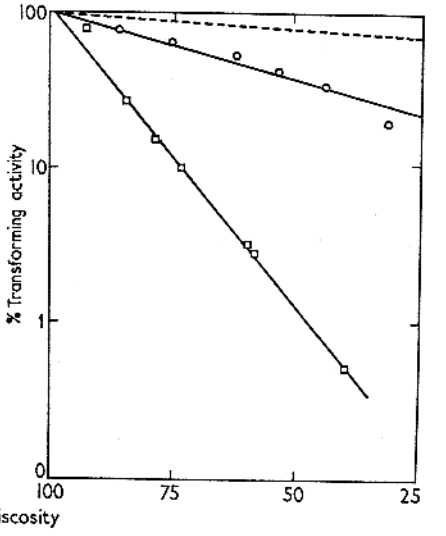

FIG. 7.

FIG. 6. Loss of competitive activity of *H. influenzae* DNA during the degradation by acid DNase at pH 5·0 (□) and by pancreatic DNase (○), plotted against viscosity.

The inhibition of transforming activity of DNA (sample E 465, erythromycin resistant) at a saturating level (1 μg/ml.) was tested at two different levels (8 and 16 μg/ml., respectively) of DNase-treated, inhibitor DNA (sample N2/A 366, cathomycin resistant). Open and filled symbols refer to the lower and to the higher concentration, respectively.

The inhibition of transforming activity by intact cathomycin DNA was proportional to the relative proportion of the latter: for example, when 8 μg of cathomycin DNA were used with 1 μg of erythromycin DNA, the transforming activity of the latter was 8 times lower than in the absence of the competing DNA. Viscosity measurements were carried out at a finite velocity gradient equal to 58 sec^{-1}.

FIG. 7. Inactivation of the cathomycin marker during the degradation of *H. influenzae* DNA (N2/A 366) by *E. coli* DNase (○) and by acid DNase at pH 5·0 (□). The broken line is an estimate of the inactivation caused by sonication obtained from data of Bach (1968).

thirty times less effective as an inactivator than the spleen enzyme. Even if our present ignorance of the *r* ratio (see Appendix) for *E. coli* DNase degradation prevents us from making the comparison in terms of breaks per DNA molecule, it is clear that a large difference exists in the inactivating abilities of the two enzymes, in spite of their very similar mechanism of action.

Sonication of transforming DNA causes diplotomic breaks and therefore an impressive decrease in molecular weight; but the inactivating effect, relative to the number of breaks, is very moderate (Fig. 7), since about 20 scissions per molecule of $M_w = 12 \times 10^6$ are needed to cause one inactivating event in the cathomycin marker (Bach, 1968). Since every scission is equal to two breaks, the total number of breaks required to cause one inactivating event in the cathomycin marker is very close to that shown by pancreatic DNase. This result is quite interesting, since Richardson (1966) has shown that sonication of phage T7 DNA produces fragments terminated by 5′ phosphates.

Both sets of results are clearly inconsistent with the idea that inactivation by acid

DNase is related to the diplotomic mechanism of action of this enzyme, and indirectly support the widely accepted view that single-stranded rather than double-stranded DNA is integrated into the host genome. It is likely that the diplotomic mechanism of degradation is responsible for the unlinking of markers found in the digestion carried out at pH 5·0.

The results just reported favour the idea that the very strong inactivation caused by acid DNase is related to the presence of 3′ phosphate ends. Concerning the mechanism by which 3′ phosphate ends hinder the integration of transforming DNA, it is conceivable that this takes place by inhibition of an enzyme involved in genetic recombination. In this connection, it is pertinent to recall the inhibition of *E. coli* DNA polymerase by templates in which 3′ phosphate ends had been introduced by partial degradation by spleen acid DNase or micrococcal nuclease (Richardson *et al.*, 1963).

We thank Drs C. Anagnostopoulos and D. Luzzati for critical reading of this paper and Miss Betty Nubel for her help with the viscosimetric measurements.

This work was aided in part by a grant from the Délégation Générale à la Recherche Scientifique et Technique.

REFERENCES

Bach, M-L. (1968). Ph.D. thesis, Strasbourg University.
Bach, M-L., Luzzati, D. & Chevallier, M. R. (1966). *Biochim. biophys. Acta,* **119**, 75.
Bernardi, G. (1964). *Makromol. Chem.* **72**, 205.
Bernardi, G. (1968*a*). *Adv. in Enzymology,* **31**, 1 (1968).
Bernardi, G. (1968*b*). *Bull. Soc. Chim. Biol.* **50**, 365 (1968).
Bernardi, G., Bernardi, A. & Chersi, A. (1966). *Biochim. biophys. Acta,* **129**, 1.
Bernardi, G. & Cordonnier, C. (1965). *J. Mol. Biol.* **11**, 141.
Bernardi, G. & Griffé, M. (1964). *Biochemistry,* **3**, 1419.
Bernardi, G. & Sadron, C. (1961). *Nature,* **191**, 809.
Bernardi, G. & Sadron, C. (1964*a*). Baselli Conference on Nucleic Acids and Their Role in Biology, Milan (Italy), September, 1963.
Bernardi, G. & Sadron, C. (1964*b*). *Biochemistry,* **3**, 1411.
Bodmer, W. F. (1966). *J. Gen. Physiol.* **49**, 233.
Cavalieri, L. F. & Rosenberg, B. H. (1961). *Biophys. J.* **1**, 323.
Charlesby, A. (1954). *Proc. Roy. Soc.* A, **224**, 120.
Cordonnier, C. & Bernardi, G. (1966). Comm. 3rd FEBS Meeting, Warsaw.
Cordonnier, C. & Bernardi, G. (1968) *Canad. J. Biochem.*, in the press.
Eigner, J. (1960). Ph.D. dissertation, Harvard University.
Eigner, J. & Doty, P. (1965). *J. Mol. Biol.* **12**, 549.
Froelich, D., Strazielle, C., Bernardi, G. & Benoit, H. (1963). *Biophys. J.* **3**, 115.
Goodgal, S. H. (1961). *J. Gen. Physiol.* **45**, 205.
Koerner, J. F. & Sinsheimer, R. L. (1957). *J. Biol. Chem.* **228**, 1049.
Kroepelin, H. (1929). *Kolloid Z.* **47**, 294.
Lehman, I. R., Roussos, G. G. & Pratt, E. A. (1962). *J. Biol. Chem.* **237**, 819.
Lerman, L. S. & Tolmach, L. J. (1957). *Biochim. biophys. Acta,* **26**, 68.
Richardson, C. C. (1966). *J. Mol. Biol.* **15**, 49.
Richardson, C. C., Schildkraut, C. L., Aposhian, H. V., Kornberg, A., Bodmer, W. F. and Lederber, J. (1963). In *Informational Macromolecules,* ed. by H. J. Vogel, V. Bryson and J. O. Lampen. New York: Academic Press.
Schaeffer, P. (1957). *C. R. Acad. Sci.* **245**, 230.
Schumaker, V. N., Richards, E. G. & Schachman, H. K. (1956). *J. Amer. Chem. Soc.* **78**, 4320.
Sevag, M. G. (1934). *Biochem. Z.* **273**, 419.
Shack, J. (1957). *J. Biol. Chem.* **226**, 573.
Sinsheimer, R. L. & Koerner, J. F. (1951). *Science,* **114**, 42.

Studier, F. W. (1965). *J. Mol. Biol.* **11**, 373.
Stuy, J. H. (1961). Ph.D. thesis, Utrecht University.
Thomas, C. A. (1956). *J. Amer. Chem. Soc.* **78**, 1861.
Young, E. T. II & Sinsheimer, R. L. (1965). *J. Biol. Chem.* **240**, 1274.
Zamenhof, S., Alexander, H. E. & Leidy, G. (1953). *J. Exp. Med.* **98**, 373.

APPENDIX

Estimation of the Ratio of Total Bonds broken to Bonds broken by Diplotomic Degradation in Native DNA digested by Spleen Acid DNase

The ratio r of *total* bonds broken to bonds broken by the diplotomic mechanism in native DNA samples digested at pH 5·0 by acid DNase may be estimated as follows.

(a) Richards & Bernardi (unpublished data, quoted by Bernardi & Sadron, 1964*a*,*b*) found, by titration, that a total of ten to twenty breaks (a value likely to be overestimated for technical reasons) was necessary to halve the weight-average molecular weight of native DNA of $M_w = 6 \times 10^6$ by spleen acid DNase digestion. Since three scissions (i.e. six phosphodiester bond breaks) are necessary to obtain this result by a diplotomic mechanism (see equation (1a) in Table 1A), an upper limit of the ratio

TABLE 1A

Degradation of DNA by acid DNase

Digestion time (min)	Native DNA $M_w \times 10^{-6}$	Native DNA Hits†	Denatured DNA $M_w \times 10^{-6}$	Denatured DNA Hits†	Hits denat. DNA‡ / Hits native DNA
Chicken erythrocytes DNA (Bernardi & Sadron, 1964*b*; Table III)					
0	3·80	—	2·20	—	—
30	2·35	1·85	1·16	2·70	1·45
60	1·75	3·51	0·75	5·82	1·66
90	1·45	4·86	0·54	9·20	1·90
Lambda phage DNA (Young & Sinsheimer, 1965; Table I)					
0	27·3	—	14·5	—	—
5	24·9	0·29	12·5	0·47	1·62
10	24·2	0·36	9·3	1·52	4·22
15	20·3	1·00	9·7	1·35	1·35
20	16·3	1·80	8·0	2·14	1·19
45	10·9	3·65	4·5	5·60	1·53

† Hits (scissions) were calculated according to Charlesby's (1954) equations:

$$\frac{(M_w)_t}{(M_w)_0} = \frac{1}{1 + \frac{1}{2}p} \tag{1a}$$

$$\frac{(M_w)_t}{(M_w)_0} = \frac{2(\exp(-p) + p - 1)}{p^2} \tag{1b}$$

Equation (1a) is valid when the initial distribution of molecular weights is the most probable one and was used in the case of chicken erythrocytes DNA. Equation (1b) is valid when the initial distribution of molecular weights is uniform and was used for calculating the data for lambda DNA. In both cases p is the average number of scissions.

‡ This column gives the ratio of total bonds broken/bonds broken by the diplotomic mechanism, per parent native molecule. In fact, scissions of denatured DNA must be multiplied by 2 to obtain the total bonds broken per parent native molecule; and scissions of native DNA must also be multiplied by 2 to obtain the number of bonds broken by the diplotomic mechanism.

r may be set at 1·7 to 3·3. It may be recalled that 200 breaks are necessary to cause the same effect when DNA is degraded by pancreatic DNase (Thomas, 1956).

(b) Bernardi & Sadron (1964*a*,*b*) measured the light-scattering molecular weights of chicken erythrocyte DNA during spleen acid DNase digestion, in both native and heat-denatured states. If one neglects thermal hydrolysis and incomplete strand separation (which compensate each other to some extent), their data can be used to calculate the ratio r, which is found to lie between 1·45 and 1·9 (Table 1A). The data suggest that the ratio tends to increase with decreasing molecular weight.

(c) Young & Sinsheimer (1965) reported weight average molecular weights calculated from zone sedimentation experiments carried out on lambda phage DNA in both native and alkali-denatured states during spleen acid DNase digestion. Table 1A shows that an r value lying between 1·2 and 1·6 can be estimated from their results, thus indicating that a small number of haplotomic breaks also take place during the degradation.

While work is in progress in our laboratory in order to get a more satisfactory estimation of the value of r, we think that the agreement of the three sets of completely independent data reported above suggests that this is of the order of 1·5 to 3·0. The ratio of haplotomic to diplotomic breaks is given by $2(r-1)$ and lies therefore between 1 and 4.