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DEGRADATION OF TRANSFORMING *HEMOPHILUS INFLUENZAE* DNA BY DEOXYRIBONUCLEASES

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SUMMARY

The inactivation of novobiocin and streptomycin resistance markers of *Hemophilus influenzae* transforming DNA by four different nucleases (spleen acid deoxyribonuclease, micrococcal nuclease, pancreatic deoxyribonuclease and *Escherichia coli* endonuclease I) has been investigated. The results obtained show that inactivation by the enzymes used is only related to the total number of breaks, and is independent of the nature of the ends formed (3′- or 5′-phosphate), and of the specificity and mechanism of action of the enzymes. Some new findings concerning the mechanism of action of the nucleases used are reported.

INTRODUCTION

Inactivation of transforming DNA by enzymatic, chemical and physical agents has been extremely useful for understanding the fundamental steps in bacterial transformation, namely DNA uptake and DNA integration into the host genome[1–10]. Inactivation by deoxyribonucleases has the advantage, over inactivation by physical and chemical agents, of being due to a single cause, namely phosphodiester bond breakage. It may be wondered, however, whether the extent of inactivation by deoxyribonucleases is exclusively related to the number of bonds broken, or if it also depends upon the mechanism of action of the enzyme (involving single and/or double breakage of native DNA), its specificity, and the nature of the ends formed (3′- or 5′-phosphate).

We present here results obtained by degrading *Hemophilus influenzae* transforming DNA with four different nucleases (spleen acid deoxyribonuclease and micrococcal nuclease, 3′-phosphate formers; pancreatic deoxyribonuclease and *Escherichia coli* endonuclease I, 5′-phosphate formers), and by correlating the inactivation of biological activity with both the total number of breaks and the number of double breaks introduced by each enzyme. The results obtained show that inactivation of two genetic markers (novobiocin and streptomycin resistance) by the deoxyribonucleases used is only related to the total number of breaks introduced by the enzymes, indepen-

dently of the nature of the ends formed and of the specificity and mechanism of action of the enzymes.

A preliminary report on part of this work was presented at the 1st European Transformation Meeting (Oeiras, Portugal, August 31st–September 2nd, 1972).

MATERIALS AND METHODS

DNA

H. influenzae DNA, preparation SN-668, was a gift of Dr M. L. Bach, who prepared it using the procedure of Bach *et al.*[11], from a mutant resistant to 5 mg/ml of streptomycin and 5 μg/ml of novobiocin (novA marker of Goodgal and Herriott[12]). The molecular weight of this DNA was estimated to be equal to $55 \cdot 10^6$, on the basis of its sedimentation coefficient. Before each enzymatic degradation, DNA was dialyzed for 20 h against the appropriate buffer: (a) 0.15 M sodium acetate buffer–0.01 M EDTA, pH 5.0 (spleen acid deoxyribonuclease); (b) 0.02 M Tris–HCl buffer, 0.005 M $MgCl_2$, pH 7.5 (*E. coli* endonuclease I); (c) 0.1 M borate buffer, pH 8.8; 0.1 M $CaCl_2$ was added to the DNA solution to a final concentration of 0.0025 M (micrococcal nuclease);(d) 0.05 M Tris–HCl buffer, pH 7.5, containing either $MgCl_2$ (0.005 M), or $MnCl_2$ (0.00125 M) (pancreatic deoxyribonuclease); in this case, DNA was previously dialyzed against 0.05 M Tris–HCl–0.01 M EDTA. The handling and dialysis of the DNA samples caused some degradation (compare the molecular weights at zero digestion time in Tables I and II with the starting one).

Enzymes

Acid deoxyribonuclease B (EC 3.1.4.6) was prepared from hog spleen[13]. A stock solution (100 units/ml) was diluted with 0.15 M acetate buffer–0.01 M EDTA, pH 5.0, in the presence of 0.05 % beef heart cytochrome *c* (Type V; Sigma, St. Louis, Mo.); the final concentration was $0.8 \cdot 10^{-4}$–$8 \cdot 10^{-4}$ units/ml.

E. coli endonuclease I (ref. 14) was purified according to a method to be described elsewhere. A stock solution (76 units/ml) was diluted with 0.02 M Tris–HCl buffer, pH 7.5, containing 0.05 % beef heart cytochrome *c*; the final concentration of enzyme was $5 \cdot 10^{-4}$ units/ml.

Micrococcal nuclease (EC 3.1.4.7) was purchased from Worthington (Freehold, N. J.; NFCP 7FA; 11 400 units/mg); the enzyme was diluted with 0.1 % bovine serum albumin (Fraction V; Sigma) in 0.1 M borate buffer, pH 8.8, to a final concentration of $5 \cdot 10^{-3}$ units/ml.

Pancreatic deoxyribonuclease (EC 3.1.4.5) was obtained from Sigma (No DN-C; lot 200-1810; 2400 Kunitz units/mg). The stock solution (1 mg/ml), stored at −70 °C, was thawed and diluted with 0.1 % bovine serum albumin in 0.05 M Tris–HCl buffer, pH 7.5. The final concentration was $6 \cdot 10^{-4}$–$100 \cdot 10^{-4}$ units/ml.

Enzymatic degradation

Degradation of native DNA (50 μg/ml) by various deoxyribonucleases was routinely followed by viscosimetry using a four-bulb capillary viscometer[15] at 28± 0.01 °C. 1-ml DNA samples were withdrawn, during degradations, added to 1 ml of cold 1 M NaCl solution and emulsified for 1 min with 0.5 ml of chloroform–isoamyl alcohol mixture (5:1, v/v) in order to inactivate enzymes. Control experiments in

which the transforming activity was assayed at different intervals of time showed that this inactivation procedure was effective.

Molecular weights and derived data

DNA molecular weights were calculated according to Studier's relationship[16] from their sedimentation coefficients, determined in neutral or alkaline solvents, using experimental conditions given elsewhere[17]. The use of the extrapolation of the Studier's relationship for alkaline s values in the low molecular weight range is essentially correct for the present purpose[17]. We will call double-stranded and single-stranded molecular weights those calculated from neutral and alkaline sedimentation coefficients, respectively.

The average number of scissions introduced by the enzymes was calculated using the equation proposed by Charlesby[18] for the case where the initital distribution of molecular weights is the most probable one:

$$\frac{M_t}{M_0} = \frac{1}{1+\frac{1}{3}p} \tag{1}$$

where p is the average number of scissions, M_0 and M_t the molecular weights at time 0 and t of digestion. It is convenient to define p_N as the number of scissions or double breaks introduced in the native DNA by the enzymes; $2\,p_A$ the total number of breaks present in the native DNA at any digestion time; $2\,p_{A_0}$ the number of single breaks (nicks) pre-existing to the enzymatic action in the starting native DNA:

$$2p_N = 6\left(\frac{M_0}{M_t}-1\right) \tag{2}$$

$$2p_A = 6\left(\frac{M_0/2}{M_t^*}-1\right) \tag{3}$$

$$2p_{A_0} = 6\left(\frac{M_0/2}{M_0^*}-1\right) \tag{4}$$

where M_0 and M_t, M_0^* and M_t^* are the molecular weights in the native and in the denatured state, respectively, at digestion times 0 and t. The p values quoted in the present work are given per 10^6 daltons of starting native DNA (samples 0 of Tables I and II). When inactivations by different enzymes were plotted against $2p_N$ or $2p_A$ values (Figs 2 and 4), these were normalized as described in the figure legends. The normalization procedure will be justified in the following section.

We define the ratio r (ref. 8) as the ratio of the total number of breaks introduced by the enzymes over twice the number of scissions (each one of them corresponding to two bonds broken) also caused by the enzymes:

$$r = \frac{2p_A - 2p_{A_0}}{2p_N} \tag{5}$$

Finally, the ratio r' of single to double breaks is given by

$$r' = 2(r-1) \tag{6}$$

Transformation

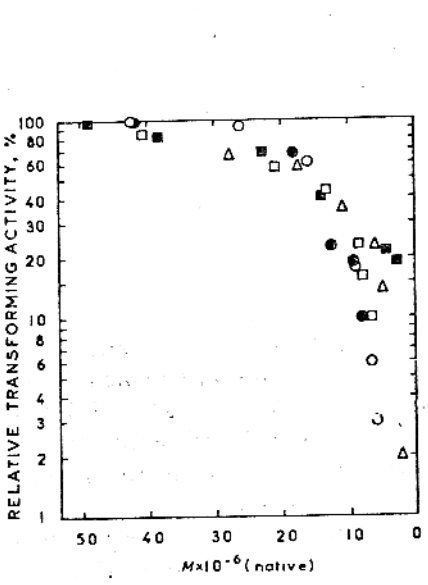
Competent cells were prepared after the Cameron modification described by Barnhart and Herriott[19]. They were stored in 15 % glycerol at −70 °C. The transforming procedure used was the overlay agar technique described by Goodgal and Herriott[12]. DNA was diluted with 0.15 M NaCl–0.02 M sodium phosphate buffer, pH 7.0, to a final concentration of 1 μg/ml. Novobiocin and streptomycin resistance were used as genetic markers.

RESULTS

Tables I and II summarize the results obtained when degradations of *H. influenzae* DNA by pancreatic deoxyribonuclease (with Mg^{2+} or Mn^{2+} as activators), spleen acid deoxyribonuclease, *E. coli* endonuclease I and micrococcal nuclease are followed by band sedimentation in neutral and alkaline solvents. Sedimentation data were used to calculate (a) double-stranded and single-stranded molecular weights; (b) the number of double and single breaks per one million of starting double-stranded molecular weight of DNA; and (c) the ratio r (see previous section). The relative transforming activities of degraded DNA are also reported in the Tables.

Correlation between inactivation and double-stranded molecular weight of degraded transforming DNA

Fig. 1 shows the residual transforming activity of the novobiocin marker



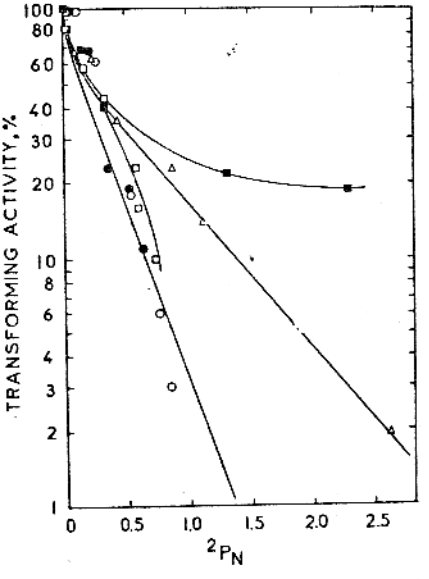


Fig. 1. Transforming activity of novobiocin marker of *H. influenzae* DNA (N column of Tables I and II) plotted against double-stranded weight-average molecular weights of samples treated by pancreatic deoxyribonuclease in presence of Mg^{2+} (○), in presence of Mn^{2+} (●), by spleen acid deoxyribonuclease (□), by *E. coli* endonuclease I (△) and by micrococcal nuclease (■).

Fig. 2. Transforming activity of novobiocin marker (N column of Tables I and II) plotted against $2p_N$. Here, $2p_N$ was calculated for all degradations, using as the starting native molecular weight that of the zero-time sample of the micrococcal series, since it had the highest native molecular weight. Symbols are those of Fig. 1.

TABLE I

DEGRADATION OF *H. INFLUENZAE* DNA BY PANCREATIC DEOXYRIBONUCLEASE

DNA sample	$s_{20,w}$ *(neutral)*	$s_{20,w}$ *(alkaline)*	*Mol. wt* $\times 10^{-6}$ *(native)*	*Mol. wt* $\times 10^{-6}$ *(denatured)*	$2p_N$ *(native)*	$2p_A$ *(denatured)*	$2p_A - 2p_{A_0}$	r	*Transforming activity (%)**		
									N	*S***	*SN***
Pancreatic deoxyribonuclease–Mg^{2+}										(43.1)	(20.6)
0	38.4	32.6	42.4	9.5	—	0.17	0	—	100	100	77
1	32.5	21.7	26.2	3.4	0.09	0.73	0.56	6.4	97	82	45
2	27.5	18.5	16.1	2.3	0.23	1.16	0.99	4.3	62	42	8.4
3	22.6	12.1	9.2	0.8	0.52	3.64	3.47	6.7	18	7	0.14
4	20.4	10.2	6.8	0.5	0.74	5.64	5.47	7.4	6	2	0.01
5	19.7	8.2	6.2	0.3	0.83	9.85	9.68	11.7	3	0.7	—
6	17.0	6.9	4.0	0.2	1.36	15.25	15.08	11.1	0.6	0.17	—
Pancreatic deoxyribonuclease–Mn^{2+}										(38.8)	(17.7)
0	38.4	36.8	42.4	12.8	—	0.09	0	—	100	91	66
1	28.8	17.8	18.4	2.1	0.18	1.29	1.20	6.5	68	45	13
2	25.3	14.9	12.7	1.3	0.33	2.10	2.01	6.1	23	15	1.4
3	22.7	13.0	9.3	1.0	0.51	3.01	2.92	5.8	19	7.9	0.56
4	21.6	11.4	8.0	0.7	0.61	4.24	4.15	6.8	11	5.4	0.16

* The zero-time (0) samples were compared with each other for the novobiocin (N) and streptomycin (S) markers and for the SN-linkage group. For both markers (N, S) and the linkage group (SN), the sample having the highest activity was taken as 100 %.

** The numbers of transformants for zero-time (0) samples relative to the highest number of transformants for the N marker (pancreatic deoxyribonuclease Mg^{2+} or Mn^{2+} series) are given in parentheses.

TABLE II

DEGRADATION OF *H. INFLUENZAE* DNA BY SPLEEN DEOXYRIBONUCLEASE, *E. COLI* ENDONUCLEASE I AND MICROCOCCAL NUCLEASE*

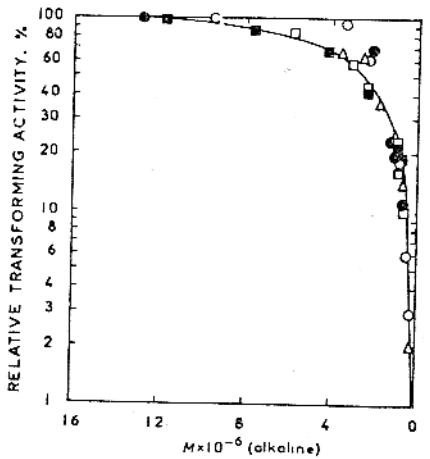
DNA sample	$s_{20,w}$ *(neutral)*	$s_{20,w}$ *(alkaline)*	*Mol. wt* $\times 10^{-6}$ *(native)*	*Mol. wt* $\times 10^{-6}$ *(denatured)*	$2p_N$ *(native)*	$2p_A$ *(denatured)*	$2p_A - 2p_{A_0}$	r	*Transforming activity (%)*		
									N	*S*	*SN*
Spleen acid deoxyribonuclease										(27.4)	(13.3)
0	37.9	27.0	40.8	5.9	—	0.36	0	—	84	64	50
1	30.2	20.8	21.2	3.1	0.14	0.83	0.47	3.5	58	51	25
2	25.7	18.2	13.3	2.2	0.30	1.22	0.86	2.9	44	25	11
3	22.1	13.1	8.6	1.0	0.55	2.95	2.59	4.7	23	12	2.1
4	20.2	10.6	8.5	0.9	0.56	3.18	2.82	5	16	9.9	0.8
5	19.2	8.8	6.8	0.6	0.73	4.85	4.5	6.18	10	4.4	0.15
E. coli endonuclease I										(25)	(5.5)
0	33.2	22.1	27.8	3.6	—	0.63	0	—	67	59	21
1	28.2	19.3	17.4	2.6	0.13	0.96	0.33	2.6	64	43	13
2	24.2	16.6	11.1	1.8	0.33	1.49	0.86	2.7	36	25	6
3	19.6	14.3	6.1	1.2	0.77	2.29	1.66	2.2	23	15	0.86
4	18.2	11.6	4.9	0.7	1.00	3.98	3.35	3.3	14	6.4	0.40
5	13.7	7.6	2.2	0.25	2.57	11.88	11.25	4.4	2	1.1	0.05
Micrococcal nuclease										(42)	(27)
0	40.4	35.6	49.0	11.8	—	0.13	0	—	99	100	100
1	37.1	29.8	38.3	7.6	0.034	0.27	0.14	4.1	83	80	53
2	31.0	23.6	22.8	4.2	0.14	0.59	0.46	3.3	69	57	28
3	25.8	18.7	13.4	2.4	0.32	1.15	1.02	3.2	41	25	7
4	17.3	13.5	4.2	1.0	1.30	2.75	2.62	2.0	22	9	0.6
5	14.5	12.0	2.5	0.8	2.27	3.73	3.60	1.6	19	6	0.3

* See footnote to Table I.

plotted against double-stranded molecular weight of *H. influenzae* DNA, as degraded by different enzymes. It can be seen that (a) the slope of the inactivation curve markedly increases below a molecular weight of about $15 \cdot 10^6$; this phenomenon seems to occur in all degradations, though less markedly in the case of micrococcal nuclease; (b) slight differences exist in the plots concerning degradations by different enzymes; such differences become quite evident if transforming activity is plotted against $2p_N$ values (Fig. 2); as judged on this basis, micrococcal nuclease is the least inactivating enzyme, followed by *E. coli* endonuclease I, spleen acid deoxyribonuclease and pancreatic deoxyribonuclease, in this order. Quite similar results were obtained in the case of the streptomycin resistance marker. The differences in the curves of Fig. 2 evidently are not related to the nature of the ends formed (3′- or 5′-phosphate) by different enzymes, a conclusion confirmed by the finding that dephosphorylation, under the conditions of Weiss *et al.*[20], of the 3′ ends formed by spleen deoxyribonuclease, and of the 5′ ends formed by *E. coli* endonuclease I in ^{32}P-labeled DNA does not lead to any change in the residual transforming activity (not shown).

Correlation between inactivation and single-stranded molecular weight of degraded transforming DNA

When the residual transforming activity of the novobiocin marker is plotted against the single-stranded molecular weight of *H. influenzae* DNA, all data practically fit the same line (Fig. 3), showing the existence of a common relationship between residual activity and single-stranded molecular weight of the DNA, regardless of the enzyme used. The slope of the inactivation curve markedly increases below a single-stranded molecular weight of $3 \cdot 10^6$–$4 \cdot 10^6$, a value which is much lower than half the value at which a similar phenomenon takes place for double-stranded mole-



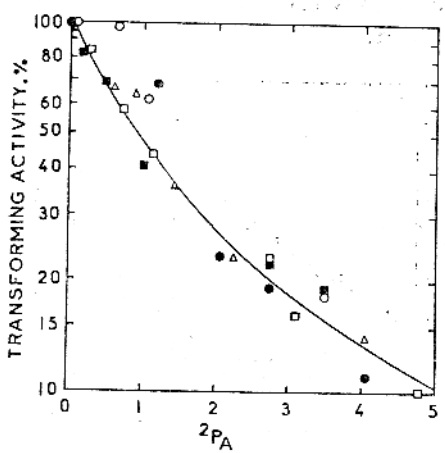


Fig. 3. Transforming activity of novobiocin marker (N column of Tables I and II) as a function of M_A determined in alkaline conditions. Symbols are those of Fig. 1.

Fig. 4. Transforming activity of novobiocin marker (N column of Tables I and II) *versus* total number of breaks, $2p_A$, introduced by the enzymes. In this case $2p_A$ was calculated, using as the starting native molecular weight the double of the single-stranded molecular weight of zero-time sample of the pancreatic deoxyribonuclease–Mn^{2+} series, which had the highest molecular weight in alkali. Symbols are those of Fig. 1.

cular weight (Fig. 1) because of the presence of single-strand breaks (nicks). This inactivation appears to be mainly due to an impaired integration of transforming DNA into the host genome, since it is well known that DNA uptake is less affected by degradation than integration[2,6–8,10,21].

Fig. 4 shows a plot of the residual transforming activity of the novobiocin marker against the total number of breaks introduced by different enzymes per one million of double-stranded molecular weight of DNA. Except for some slight trends of doubtful significance, all points fall on the same line, regardless of the enzyme used. Curves similar to those of Figs 3 and 4 were obtained with the streptomycin resistance marker (not shown).

It should be pointed out that the total number of breaks in Fig. 4 have been normalized by taking the highest zero-time single-stranded molecular weight DNA as the reference DNA for all enzymes. In this way, each $2p_A$ value in Fig. 4 corresponds to the same single-stranded molecular weight for all enzymes. This procedure implies that the breaks introduced by handling and dialyzing the DNA samples are as inactivating as those introduced by the enzymes. Such assumption is justified, *a posteriori*, by the finding that different enzymatic activities have the same inactivating effect, as judged on the basis of the total number of breaks introduced. A similar procedure was applied for the data of Fig. 2.

Figs 5a and 5b show the plots (N and S) of the reciprocal of the square root of the transforming activity of novobiocin and streptomycin markers, respectively, *versus* the reciprocal of the single-stranded molecular weight of degraded DNA samples according to Bresler *et al.*[22]. For both markers, data obtained in all enzymatic degradations fit the same straight line. According to Bresler *et al.*[22], the slope of these lines is equal to $1/2\ \omega$ where $1/\omega$ is the size of the genetic unit, expressed here in daltons of double-stranded DNA. We obtain, by a least-squares method, $2.9 \cdot 10^6$ and $5.3 \cdot 10^6$ daltons, respectively, for the size of the genetic unit of novobiocin and streptomycin markers.

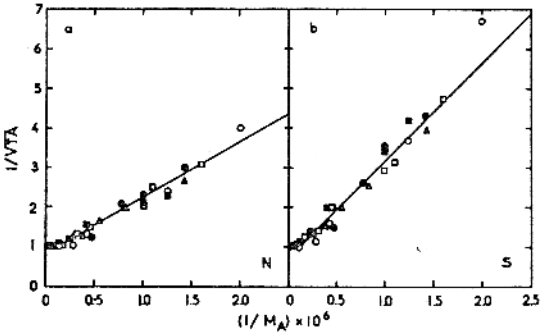


Fig. 5. Plot of the reciprocal of the square root of transforming activity (TA) of novobiocin marker (N column of Tables I and II) (a) and of streptomycin marker (S column of Tables I and II) (b) *versus* the reciprocal of the alkaline molecular weight, according to Bresler *et al.*[22]. Symbols are those of Fig. 1.

Fig. 6 shows the same plot as in Fig. 5 for the linkage group SN. Data for all enzymes again fit the same curve, which is slightly above the parabolic curve (dotted line) corresponding to the product of the two straight lines N and S.

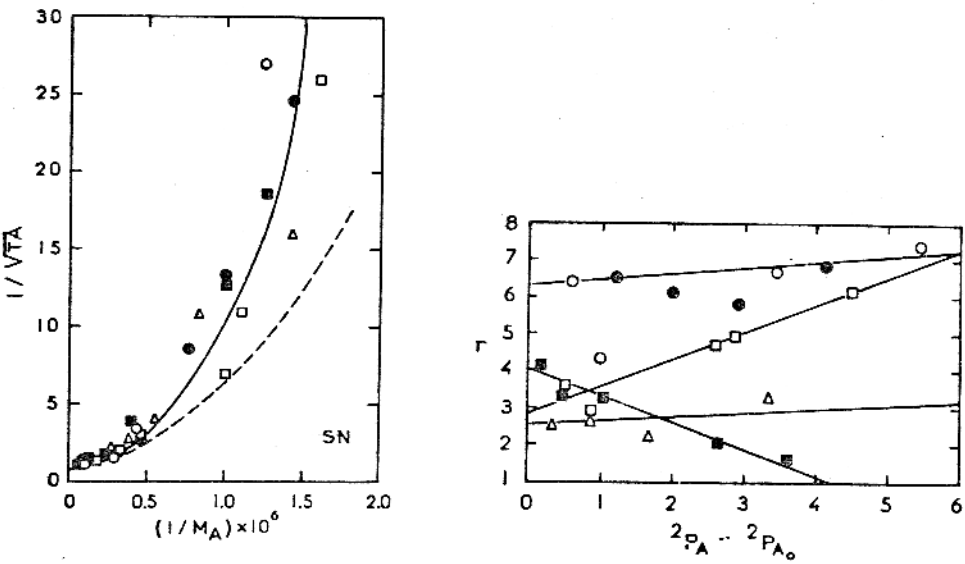


Fig. 6. Plot according to Bresler *et al.*[22] (see Fig. 5) for the SN-linkage group (SN column of Tables I and II). The dashed curve corresponds to the product of the two transforming activities (TA) of N and S markers as given by the average straight lines of Figs 5a and 5b.

Fig. 7. Plot of the r ratio against the total number of breaks ($2p_A$–$2p_A$ values from Tables I and II) introduced in *H. influenzae* DNA by different deoxyribonucleases. Symbols are those of Fig. 1.

Mechanisms of degradation of DNA by different deoxyribonucleases

Fig. 7 shows a plot of the r ratio against the total number of breaks introduced by the enzymes used in the present work, per one million of double-stranded molecular weight of DNA. It is evident that the r ratio increases as digestion proceeds in the case of acid deoxyribonuclease, whereas it decreases in that of micrococcal nuclease. The r ratio seems to show no increase or a slight increase in the case of pancreatic deoxyribonuclease and in that of *E. coli* endonuclease I. The intercepts at the origin on the r axis are 2.8, 2.5 and 4.0 for spleen acid deoxyribonuclease, endonuclease I and micrococcal nuclease, and about 6.5 for pancreatic deoxyribonuclease. These values correspond to r' ratios of 3.6, 3.0, 6.0 and 11.0, respectively.

DISCUSSION

The main conclusion which can be drawn from the present investigations is that the inactivation of transforming DNA by the different nucleases investigated is only related to the total number of breaks introduced by the enzymes; in fact no difference can be observed when inactivation is plotted against single-stranded molecular weight (Figs 3, 5 and 6) or total number of breaks (Fig. 4). This conclusion confirms and extends the original proposal of Bodmer[6] for *Bacillus subtilis* DNA inactivation and the results of Thorsett and Hutchinson[10] on the degradation of *Diplococcus pneumoniae* DNA by pancreatic deoxyribonuclease and fits in with the accepted view that single-stranded transforming DNA is integrated into the host genome[5,23,24]. This conclusion implies that inactivation is independent (a) of the nature of the ends formed (3'- or 5'-phosphate), a fact also shown by direct dephos-

phorylation experiments, (b) of the mechanism of action of the enzymes used, as judged from the ratio *r*, and (c) of their specificity. Since the enzymes used have quite different specificities and since it is well established that large compositional heterogeneities in DNAs can be revealed by enzymes like the spleen and micrococcal nucleases (ref. 25 and Prunell, A. and Bernardi, G., unpublished), this means that the nucleotide sequences recognized by them have essentially the same frequency in the genetic markers under consideration and in the region between them as in the total *H. influenzae* DNA.

These results imply very similar single-stranded molecular weight distributions in the low molecular weight range for DNA samples having the same alkaline *s* values, as obtained by degradation with different enzymes. In fact, differences in the distribution curves would lead to different transforming activities because of the strong dependence of the latter upon single-stranded molecular weight below a value of $3 \cdot 10^6$ daltons. This observation is in keeping with a size distribution corresponding to the most probable one (a fact already observed for spleen acid deoxyribonuclease digests[26]) and, therefore, with a random distribution of breaks in all degradations.

Concerning the inactivation of the streptomycin/novobiocin (SN)-linkage group, no differences were found in degradations caused by different enzymes. Moreover, an identical extent of unlinking of the two genetic markers is indicated by the displacement of the experimental curve compared to the one corresponding to the product of the inactivation curves of the two separate markers (Fig. 6).

The data obtained on native DNA (Figs 1 and 2) are interesting in that differences are seen in degradations by different enzymes. Such differences are clearly related to the different mechanisms of action as judged by the *r* values of different deoxyribonucleases. For instance, pancreatic deoxyribonuclease (with both metal ions) is more inactivating than *E. coli* endonuclease I at comparable $2p_N$ levels because of its higher *r* value (see Tables I and II and below).

The results of Fig. 7 show several features of enzymological interest, independent of their bearing on the main issue investigated here. These can be briefly summarized as follows.

(a) Spleen acid deoxyribonuclease. The present estimate of the *r* ratio for this enzyme (as extrapolated to zero digestion time) is close to 2.5, in fair agreement with the preliminary estimate, 1.5–3, given by Bernardi and Bach[8]. The increase in the *r* ratio with degradation extent is also in agreement with preliminary observations by Bernardi and Bach[8] and with later unpublished results of our laboratory; the explanation given for this phenomenon (Soave, C., Thiery, J. P., Ehrlich, S. D. and Bernardi G., unpublished) is that this enzyme, which is known to attack two different kinds of sites, the first one by a double breakage, the second one by a single breakage mechanism[27], rapidly exhausts the first kind of sites, which are limited in number.

(b) Micrococcal nuclease, in contrast, shows a decrease in its *r* ratio as digestion proceeds. This enzyme is known to have a strong preference for AT-rich regions in DNA and tends therefore to accumulate single breaks in these regions (ref. 28 and Prunell, A. and Bernardi, G. unpublished). It is conceivable, therefore, that less and less new single breaks are needed to cause scissions in double-stranded DNA, as digestion proceeds. It should be stressed that in this case the number of breaks, as calculated from sedimentation measurements, will tend to be underestimated, because scissions will, in general, be due to more than two breaks on the opposite strands; in

addition the exonucleolytic breaks caused by the enzyme will be neglected.

(c) *E. coli* endonuclease I does not show a significant trend in its *r* value; this implies the existence of a constant ratio between the number of breaks leading to scissions and that of breaks which do not cause scissions.

(d) Pancreatic deoxyribonuclease. In this case, too, *r* tends to remain constant. Interestingly, no significant difference in the *r* ratio was found according to whether Mg^{2+} or Mn^{2+} were used as activators; this result is rather surprising in view of the report that the enzyme causes double breaks in the presence of Mn^{2+} and single breaks in the presence of Mg^{2+} (refs 29, 30). It should be noted that only a very slight change in specificity was found when using Mn^{2+} instead of Mg^{2+} (Ehrlich, S. D., Bertazzoni, U. and Bernardi, G., unpublished). The ratio *r* found here for pancreatic deoxyribonuclease is close to 6, a value within the range (3–18) g'ven by Laipis *et al.*[30], but much lower than those estimated by previous authors (see ref. 31, and Schumaker, V. N., Richards, E. G. and Schachman, H. K., unpublished data quoted in ref. 8) and used by Bernardi and Bach[8], who were so misled into concluding that spleen acid deoxyribonuclease is more inactivating than pancreatic deoxyribonuclease. A final point which should be made concerning pancreatic deoxyribonuclease is that no evidence of a lag time was found in the present work. Investigations in progress on several of these points will be presented elsewhere in due time.

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