*J. Mol. Biol.* (1962) **5**, 424–434

# The Structure of the DNA of Bacteriophage ϕX174

## III. Ultracentrifugal Evidence for a Ring Structure

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(*Received 20 June 1962*)

By velocity sedimentation, in appropriate solvents, the presence of two discrete components can be demonstrated in preparations of the DNA of bacteriophage $\phi$X174. The major, faster-moving $S_1$ and the slower $S_2$ are present under conditions which exclude the possibility of hydrogen-bond formation. It can be shown, either by treatment with pancreatic deoxyribonuclease or by thermal inactivation, that $S_2$ is the first degradation product of $S_1$, formed by scission of $S_1$, without significant decrease in molecular weight. A subsequent chain scission in $S_2$, which occurs with equal likelihood, results in random fragmentation. These results are interpreted to mean that the $S_1$ component is a covalently linked ring structure and the $S_2$ component is the corresponding open-chain degradation product.

Under certain conditions the $S_2$ component can be selectively degraded by *E. coli* phosphodiesterase. The digestion is not complete and there appears to be a single discontinuity, resistant to phosphodiesterase, present in the $\phi$X-DNA ring.

## 1. Introduction

One of us (Sinsheimer, 1959) has reported that when $\phi$X DNA was centrifuged under conditions which produced an extension of the molecules, two components could be demonstrated. The minor component (henceforth called $S_2$) comprised about 20 to 40 % of the total, and under the conditions employed, moved at about a 10% slower rate than the major component ($S_1$), which usually comprised 50 to 70%. This heterogeneity was rather surprising in a preparation derived from a supposedly homogeneous virus preparation. The difference was suspected to be configurational, as the separation was not observed during centrifugation in a 0·2 M-salt solution, and because the nucleotide composition seemed to be independent of the relative amounts of $S_1$ and $S_2$.

In a previous paper (Fiers & Sinsheimer, 1962*a*) evidence has been presented which indicates that infective $\phi$X-DNA molecules lack both a free 3′-OH and a free 5′-OH terminus. The following relation explains both this chemical peculiarity and the physical duality: the $S_1$ seems to be identical with the infective form and has a ring structure, while $S_2$ is the first breakdown product of $S_1$ and corresponds (after treatment with formaldehyde, or in alkali) to an open chain form.

## 2. Materials and Methods

The sources of the materials have been described in a previous paper (Fiers & Sinsheimer, 1962*a*).

The "standard treatment" with formaldehyde consisted of adding 1/19 vol. of 36% formaldehyde (Baker and Adamson, reagent grade) followed by 20 hr incubation at 37°C.

Centrifugation was performed in the Spinco model E ultracentrifuge. Alkaline solutions were centrifuged in Kel-F centerpieces.

Quantitation of ultracentrifuge patterns was routinely performed by means of the reference-wedge method of Robkin, Meselson & Vinograd (1959). No discrepancy was found between these results and results obtained by direct evaluation from the densitometer tracings. This indicates that the film response was linear with DNA concentration, and that the photographic procedure was reproducible. Values reported are the averages from two frames, usually the no. 5 frame (40 min) and the no. 7 frame (56 min).

In the digestions with pancreatic DNase (Fig. 2) each enzymic reaction mixture (200 $\mu$l.) contained 30 $\mu$g $\phi$X DNA (preparation 6), $1{\cdot}25 \times 10^{-3}$ M-tris buffer, pH 7·5, $5 \times 10^{-3}$ M-$MgCl_2$, $5 \times 10^{-2}$ M-NaCl, $5 \times 10^{-3}$% BSA,† and $7 \times 10^{-5}$ $\mu$g pancreatic DNase. To terminate the reaction the digest was diluted fivefold with potassium phosphate buffer and EDTA to yield final concentrations respectively of 0·08 M and 0·002 M at pH 9·0. A sample was taken for assay of the biological activity and the remainder was analysed in the ultracentrifuge after the standard treatment with formaldehyde.

For the thermal inactivation study (Fig. 3), each sample (200 $\mu$l.) containing 34 $\mu$g $\phi$X DNA (preparation 5), 0·025 M-glycine buffer pH 9·0, $5 \times 10^{-5}$ M-EDTA, and 0·025 M-NaCl, was heated at 98·2°C for a specified time and fast-cooled. A sample was taken for assay of biological activity and the remainder was analysed in the ultracentrifuge after the standard treatment with formaldehyde.

The combined digestions of $\phi$X DNA with pancreatic DNase and *E. coli* phosphodiesterase (Fig. 5) were performed as follows: a 50 $\mu$l. reaction mixture containing 52 $\mu$g $\phi$X DNA (preparation 3), 0·1 M-NaCl, 0·0021 M-tris buffer pH 7·5, 0·008 M-$MgCl_2$, 0·01% BSA, and 62 $\mu\mu$g pancreatic DNase was incubated at 37°C for an appropriate time. 100 $\mu$l. 0·05 M-sodium carbonate buffer, pH 11·0, was added and the sample incubated 2 hr at 30°C. The sample was then diluted to 300 $\mu$l. by addition of 4·5 $\mu$moles of HCl–glycine buffer, pH 9·5, to a final concentration of 0·066 M and 50 $\mu$l. of phosphodiesterase. After 24 hr incubation at 30°C a 100 $\mu$l. sample was taken for measurement of per cent hydrolysis (to acid solubility) while the remainder was used for assay of biological activity and for ultracentrifugal analysis (Table 6).

## 3. Results

### (a) *Observation of sedimentation heterogeneity in $\phi$X DNA*

In the earlier study, heterogeneity of $\phi$X DNA was observed during sedimentation in formaldehyde, or in a medium of low ionic strength. In the former medium, $\phi$X DNA is irreversibly inactivated; in the latter its biological activity is unstable and reproducible observation of heterogeneity is difficult, probably because of convection during sedimentation.

A quantitatively similar pattern can be obtained during sedimentation in alkaline medium at pH 11·0 (Table 1, experiment 1). $\phi$X DNA can be recovered from this medium with full biological activity (after 2 hours at 30°C in 0·033 M-sodium carbonate, pH 11·0, plus 0·01 M-NaCl, plus $1{\cdot}3 \times 10^{-4}$ M-EDTA).

The $S_1$ and $S_2$ components are also present during sedimentation at pH 12·0 (Table 1, experiment 2) and at pH 12·5, in the usual proportions. As pH 12·5 is well above the denaturation point of any DNA studied (Vinograd, Morris, Dove & Davidson at the sixth annual meeting of the American Biophysical Society, 1962; Dove 1962), this observation would seem to exclude the possibility that the distinction between $S_1$ and $S_2$ is produced by a hydrogen-bonded configuration. This conclusion is supported by the persistence of the $S_1$, $S_2$ pattern after heating of the $\phi$X DNA to 80°C in formaldehyde (Table 1, experiment 3), a condition known to denature calf thymus DNA irreversibly.

† Bovine serum albumin.

TABLE 1

*Sedimentation heterogeneity of $\phi$X DNA*

| Experiment | Conditions of centrifugation | Sedimentation coefficient | | % Composition | | |
|---|---|---|---|---|---|---|
| | | $S_1$ | $S_2$ | $S_1$ | $S_2$ | Slow† |
| 1‡ | $CH_2O$ (control) | 13·5 s | 12·1 s | 72·2 | 18·9 | 8·9 |
| | $OH^-$ (pH 11) | 12·8 | 12·1 | 67·7 | 16·8 | 15·5 |
| | $OH^-$ (pH 11) | 13·1 | 12·0 | 74·4 | 20·4 | 9·2 |
| 2§ | $OH^-$ (pH 12) | 11·5 | 10·7 | 62·4 | 25·7 | 11·9 |
| 3‖ | $CH_2O$ (control) | 13·1 | 12·1 | 60·4 | 17·0 | 22·6 |
| | $CH_2O$ (heated) | 13·8 | 12·3 | 57·0 | 25·3 | 17·7 |

† All centrifuge patterns contain a certain amount of slower moving degradation products with a distribution of sedimentation coefficients.

‡ The control contained 32 $\mu$g $\phi$X DNA (preparation 6) in 1 ml. 0·1 M-sodium phosphate buffer, pH 7·0 plus 0·01 M-NaCl. It was treated with formaldehyde as described. The pH 11 experiments contained 30 $\mu$g $\phi$X DNA (preparation 6) in 1 ml. 0·04 M-sodium carbonate buffer, pH 11·0 plus $6 \times 10^{-4}$ M-EDTA plus 0·01 M-NaCl.

§ The pH 12 experiment contained 30 $\mu$g $\phi$X DNA (preparation 6) in 0·032 M-sodium arsenate buffer, pH 12·0.

‖ Control, as in experiment 1, but with $\phi$X DNA preparation 3. The heated sample was held at 80°C for 10 min in the presence of the formaldehyde, fast-cooled, and centrifuged.

### (b) *Correlation of biological activity and $S_1$ content*

In the course of various treatments of $\phi$X DNA (Fiers & Sinsheimer, 1962*a*,*b*) it was observed that after exposure to enzyme preparations which presumably produced chain scission, no quantitative correlation could be made between the residual biological activity and the proportion of the total DNA in the leading boundary when ultracentrifugal analysis was performed in 0·1 to 0·2 M-salt solution (Table 2).

TABLE 2

*Ultracentrifuge patterns of $\phi$X DNA partially inactivated by various endonucleases*

| Enzyme | Residual biological activity (%) | Solvent for ultra-centrifugation | Proportion in principal boundary (% of control) | Calculated† proportion of $S_1$ plus $S_2$ (%) |
|---|---|---|---|---|
| *E. coli* phosphatase | 42 | A | 62·8 | 78·4 |
| Pancreatic DNase | 37 | B | 80·1 | 74·0 |
| Venom diesterase | 17 | A | 49·8 | 43·5 |
| Spleen diesterase | 1·4 | C | 0 | 7·5 |

Solvents: A is 0·1 M-sodium phosphate, pH 7·0 plus 0·002 M-EDTA.
B is 0·22 M-NaCl plus 0·01 M-tris, pH 8·1.
C is 0·08 M-potassium phosphate, pH 9·0.

† Calculated from residual biological activity assuming a Poisson distribution of "hits".

However, sedimentation of several variously treated $\phi$X DNA preparations under conditions which permitted observation of sedimentation heterogeneity demonstrated a direct correlation between infectivity and the content of the $S_1$ component (Fig. 1).

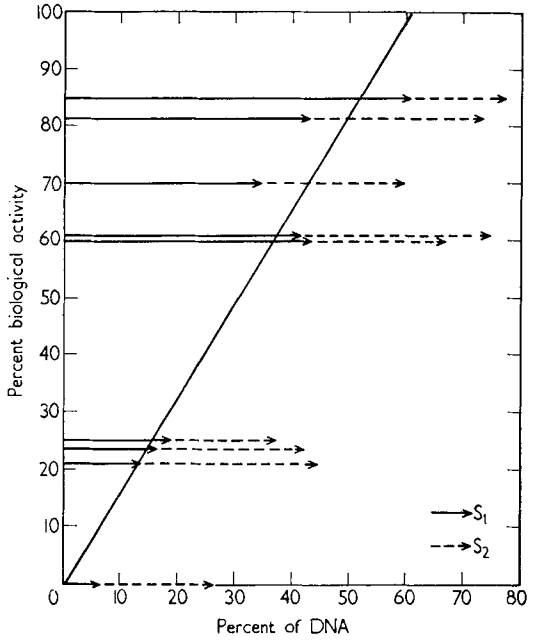



FIG. 1. Correlation between biological activity and ultracentrifugal components. Results of samples with different histories are collected. Treatments include enzymic, thermal and spontaneous inactivation. The ordinate is the percentage residual biological activity, compared to a standard $\phi$X DNA (preparation 3) of equal ultraviolet absorption.

### (c) *Effect of action of pancreatic DNase upon infectivity and sedimentation pattern of $\phi$X DNA*

If $\phi$X DNA is treated with pancreatic DNase for various periods of time, it is found (Fig. 2) that the proportion of the $S_1$ component decreases exponentially, as would be expected for the infective component (Guthrie & Sinsheimer, 1960). Using the residual biological activity to calculate the number of DNase "hits" upon $S_1$, it is found that the proportion of $S_2$ follows closely the expected function for the proportion of the component with one hit. First the proportion of $S_2$ rises to nearly the theoretical value (37% at one hit), indicating that it is formed from $S_1$ without appreciable loss of substance or decrease in molecular weight (excluding a specific, median scission, *vide infra*). After an exposure to the enzyme sufficient to produce an average of one hit per molecule, the proportion of $S_2$ decreases, always remaining larger, however, than the proportion of $S_1$. The good agreement between the experimental points and the calculated function also indicates that the probability of destruction of $S_2$ (by a second hit) is similar to the probability of formation of $S_2$ from $S_1$ by the first hit (thus excluding the possibility of a special site of attack for conversion of $S_1$ to $S_2$). No discrete boundary other than $S_1$ and $S_2$ was observed in any of these experiments. A few samples, centrifuged in alkali rather than in formaldehyde, gave quantitatively similar results.

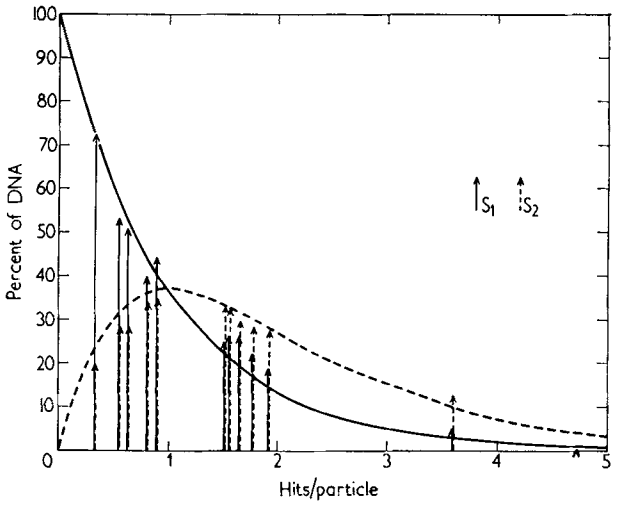



FIG. 2. Ultracentrifugal composition of $\phi$X DNA after varying extent of hydrolysis by pancreatic DNase. The ordinate is the proportion of ultraviolet absorption contributed by $S_1$ and $S_2$ to the ultracentrifuge pattern. The abscissa is the number of "hits" calculated from the residual biological activity. The control (no enzyme added) was considered to contain 72% (the same as its $S_1$ content) of the biological activity of a theoretical fully-active sample (i.e. we assume 28% of the DNA has already received one or more "hits" during its preparation).

The smooth curve is the theoretical function for the no-hit ($e^{-m}$, where $m$ is the mean number of hits per molecule) fraction. The dashed curve corresponds to the one-hit function ($m.\ e^{-m}$).

The sedimentation coefficients of the $S_1$ component were between 13·5 and 14·1 s: of $S_2$ component between 11·5 and 12·5 s.

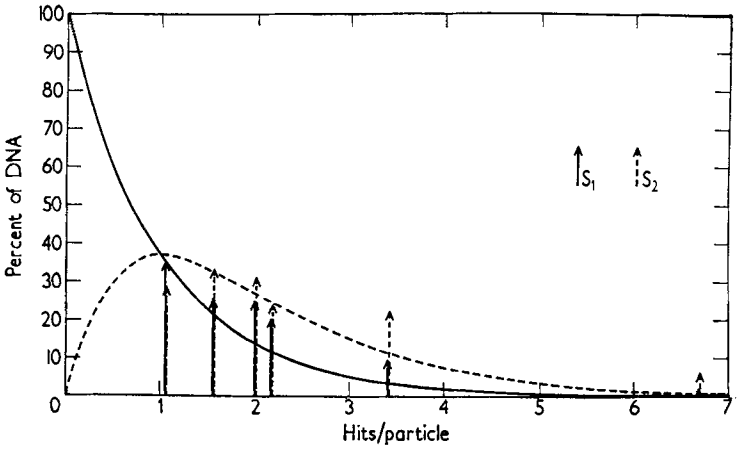



FIG. 3. Ultracentrifugal composition of $\phi$X DNA after varying extent of thermal inactivation. Ordinate, abscissa and curves as in Fig. 2. The $S_1$ content of the unheated control was 35% for this preparation.

The sedimentation coefficients of the $S_1$ component were between 12·8 and 13·2 s; of the $S_2$ component between 11·2 and 11·5 s.

(d) *Effect of heat upon infectivity and sedimentation pattern of $\phi$X DNA*

A similar pattern of centrifugal observations is obtained (Fig. 3) if inactivation of $\phi$X DNA is brought about by heat rather than by enzymic action. The results in Fig. 3 are somewhat more erratic and in poorer agreement with the theoretical functions. The proportions of the $S_1$ and $S_2$ components exceed those expected from the degree of inactivation. However, it is evident that the application of heat, as that of DNase, results in the conversion of $S_1$ to $S_2$ and in the degradation of $S_2$. The chance of the second event is comparable with the chance of the first, thus indicating that there is no especially heat-sensitive site for chain scission.

(e) *Masked or potential chain scission in $\phi$X-DNA structure*

The results just described indicate that thermal inactivation (at pH 9·0) can result in a conversion of $S_1$ to $S_2$ (and further) and thus by analogy with the results obtained with DNase, can be accompanied by chain scission. However, in a previous paper (Fiers & Sinsheimer, 1962*b*) we have demonstrated that thermal inactivation of $\phi$X DNA is not immediately accompanied by a susceptibility to the action of *E. coli* phosphodiesterase. Further centrifugal analyses, under conditions which do not rupture hydrogen bonds, of $\phi$X-DNA preparations inactivated by heat or acid (Table 3) do not reveal any simple correlation between extent of inactivation and that of molecular degradation. When the same preparations are centrifuged in the presence of formaldehyde, a distinctly lesser, but still unexpectedly large amount of macromolecular components is observed.

TABLE 3

*Ultracentrifuge patterns of variously inactivated $\phi$X DNA*

| Treatment | Residual biological activity (%) | Proportion in leading boundary† (% of control) | Calculated¶ proportion of $S_1$ plus $S_2$ (%) | Observed proportion of $S_1$ plus $S_2$ in formaldehyde (%) |
|---|---|---|---|---|
| Heat‡ | <0·1 | 56·7 | <0·8 | 22·6 |
| pH 2·9§ | 0·03 | 104·5 | 0·3 | 28·3 |
| Isobutanol‖ | 23·5 | 100·6 | 57·6 | 47·5 |
| Isobutanol | 20·9 | 101·2 | 53·7 | 51·6 |

† Centrifuged in 0·1 M-sodium phosphate plus 0·002 M-EDTA.
‡ 10 min at 100°C, at pH 7·5.
§ 15 hr in 0·1 N-acetic acid at 30°C.
‖ Repeated extraction of solution with isobutanol as described in Fiers & Sinsheimer (1962*a*).
¶ Calculated from residual biological activity, assuming a Poisson distribution of hits.

Evidently the inactivating event upon exposure of $\phi$X DNA to either heat or acid can leave the macromolecular structure intact. Some degree of chain scission can, however, be demonstrated after exposure of the inactivated DNA to more disruptive conditions (higher temperature, formaldehyde, alkali). Either some chain scission has occurred during inactivation and its effect is masked by hydrogen-bonding of adjacent nucleotides† or the chain scission subsequently observed is not an immediate

† A masking of scissions in this way does not seem to occur if the scissions are produced by endonuclease action (Table 2). Following endonuclease action the proportion of DNA in fragments of $S$ less than that of the principal component is very close to that expected from the number of hits.

consequence of the inactivating event (presumably depurination) but occurs at the weakened site upon molecular extension. The persistence of macromolecular components (Fig. 3, Table 3) in amounts considerably in excess of those to be expected from the number of hits, suggests that scission does not occur at all such sites under conditions employed here.

The nature of inactivation by extraction with isobutanol is unknown, but evidently the process results in direct or potential chain scissions which are quantitatively revealed in the presence of formaldehyde.

### (f) *The endogenous $S_2$ component*

All $\phi$X-DNA preparations thus far examined contained proportions of $S_1$, $S_2$ and slower moving fragments which approximately correspond to the proportions to be expected of zero, one, and more-than-one hit components in a population which has been subjected to random degradation (Table 4). The nature of these hits may be heterogeneous (nucleolytic, depurination) and may vary from one preparation to another.

TABLE 4

*Sedimentation heterogeneity of $\phi$X-DNA preparations*

| Preparation | $\%S_1$ | $\%S_2$ | | % Slow component | |
|---|---|---|---|---|---|
| | | Found | Expected | Found | Expected |
| 3 | 64·5 | 19·7 | 28·3 | 15·8 | 7·2 |
| 5 | 36·3 | 34·2 | 36·8 | 29·5 | 26·8 |
| 6 | 72·2 | 18·9 | 23·5 | 8·8 | 4·3 |

Each preparation was analysed after formaldehyde treatment. Expected amounts of $S_2$ (one-hit) and slow (more-than-one-hit) components were calculated from the proportion of the $S_1$ (zero-hit) component.

In one preparation (preparation 6) the $S_2$ component could be completely degraded by the action of *E. coli* phosphodiesterase (Fig. 4). However, the $S_2$ component of other preparations has been largely resistant to *E. coli* phosphodiesterase attack (Table 5). Prior treatment with *E. coli* phosphomonoesterase, while resulting in some degradation because of endonuclease action, did not significantly increase the susceptibility of $S_2$ to *E. coli* phosphodiesterase action.

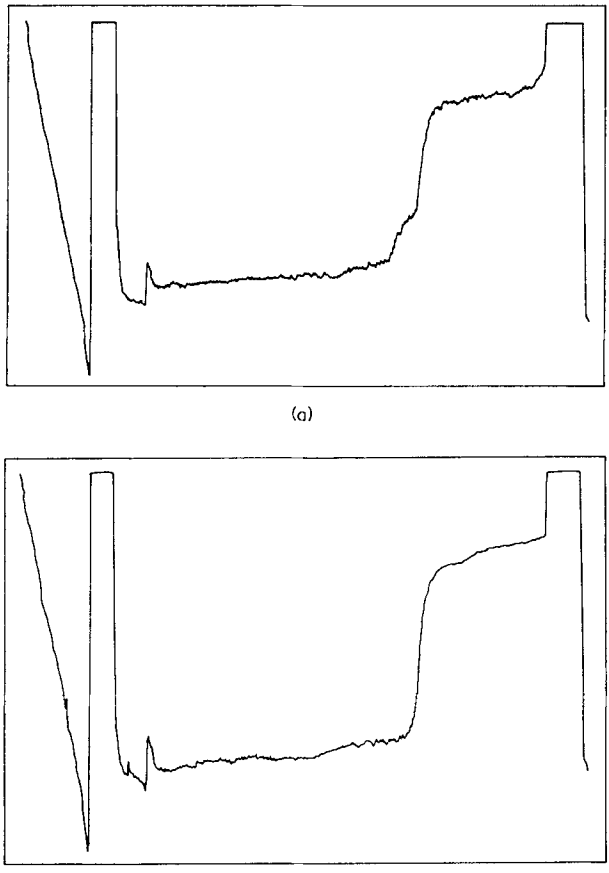
The possibility that the termini of the $S_2$ component molecules could be protected from phosphodiesterase attack by coiling and hydrogen bond formation would seem to be excluded by the evident susceptibility of $S_2$ component formed by pancreatic DNase action (cf. Fig. 5 of this paper and Fig. 3 of Fiers & Sinsheimer, 1962*a*). A more plausible explanation of the resistance of endogenous $S_2$ component is that—by analogy to the depurinated molecules described above—it remains in a ring form until exposed to conditions producing molecular extension. If this is so, a prior exposure to such conditions (e.g. alkali) should render at least a portion of the potential $S_2$ component susceptible to phosphodiesterase action. Preliminary experiments indicate that this hypothesis is correct.

TABLE 5

*Effect of* E. coli *phosphodiesterase upon sedimentation pattern of $\phi$X DNA*

| % Hydrolysis† (acid soluble) | % Composition | | | |
|---|---|---|---|---|
| | $S_1$ | $S_2$ | Slow | Total |
| 0 (control) | 48·2 | 30·7 | 20·1 | 100 |
| 12·5 | 45·4 | 25·6 | 10·6 | 81·6 |
| 0 (control) | 81·8 ($S_1+S_2$) | | 18·1 | 100 |
| 16·1 | 72·4 ($S_1+S_2$) | | 8·0 | 80·4 |

† Experimental procedure identical to that described in Fig. 2, Fiers & Sinsheimer (1962*a*).



(a)

(b)

FIG. 4. Selective degradation of the $S_2$ component by *E. coli* phosphodiesterase. Each sample contained 30 $\mu$g/ml. $\phi$X DNA (preparation 6). Sedimentation from left to right at 56,100 rev./min. Pictures taken after 56 min of centrifugation. Densitometer traces from Joyce-Loebl densitometer.

(a) Control, centrifuged in 0·04 M-sodium carbonate buffer, pH 11·0 plus 0·01 M-NaCl plus $1{\cdot}5 \times 10^{-4}$ M-EDTA.

(b) The sample was pre-incubated for 2 hr at 30°C in the solvent described in (a). Glycine buffer, pH 9·5, $MgCl_2$, and *E. coli* phosphodiesterase (final concentration, 166 $\mu$l./ml.) were added and digestion carried out for 5 hr at 37°C. After addition of EDTA to halt reaction, the sample was dialysed against the carbonate buffer solvent described in (a) and then analysed in the ultracentrifuge.

### (g) *A discontinuity in the φX-DNA ring*

The extent of hydrolysis possible by *E. coli* phosphodiesterase after varying degrees of pancreatic DNase action can be measured both by determination of the amount of acid-soluble nucleotides produced and by measurement of the decline in the ultraviolet absorbing sedimentable material in the ultracentrifuge. The results of both measurements (Table 6) are in good agreement. When the data are plotted (Fig. 5)

TABLE 6

| Biological inactivation (hits/molecule) | % hydrolysis (acid soluble) | Sedimentation analysis | |
|---|---|---|---|
| | | Hits/molecule† | % hydrolysis‡ |
| 0 (control)§ | 0 | 0 | 0 |
| 0 (control)§ | 0 | 0 | 0 |
| 0·20 | 20·0 | 0·43 | 14·3 |
| 1·2 | 38·0 | 1·2 | 33·9 |
| 1·4 | 48·4 | 1·5 | 38·9 |
| 2·2 | 55·0 | 1·8 | 46·5 |
| 3·8 | 75·1 | >2 | 63·0 |
| 4·9 | 79·0 | >2 | 69·4 |

Biological activity, percent hydrolysis, and sedimentation data after successive action of pancreatic DNase and *E. coli* phosphodiesterase, as in Fig. 5. For sedimentation, the 200 μl. reaction mixture was diluted fivefold with sodium carbonate and EDTA (final concentrations 0·04 M and $6{\cdot}7 \times 10^{-4}$ M respectively) and 5 μmole NaOH added to adjust the pH to 11·0.

† Calculated from decrease in $S_1$.

‡ Decrease in sedimentable, ultraviolet absorbing material.

§ Recovery of biological activity in the controls was 109 and 95%, respectively.

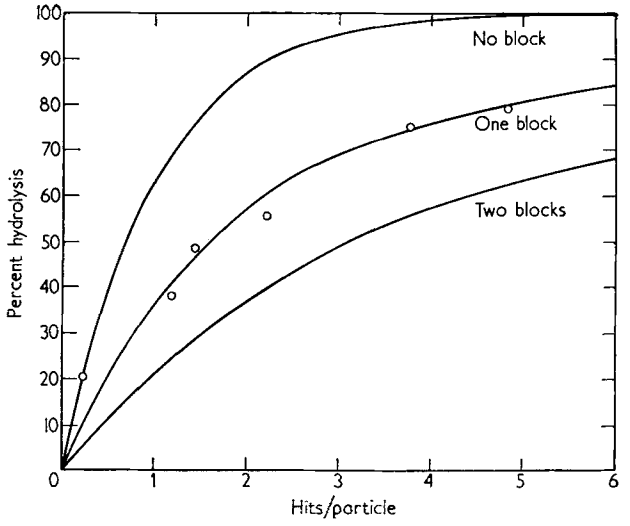



FIG. 5. Extent of hydrolysis by *E. coli* phosphodiesterase following limited digestion by pancreatic DNase. The ordinate is the percentage of φX DNA hydrolysed, as measured by the amount of acid-soluble ultraviolet absorption, expressed relative to the ultraviolet absorption of the non-hydrolysable material of the control (φX DNA treated with *E. coli* diesterase, but not pretreated with pancreatic DNase). The abscissa is the number of "hits" due to pancreatic DNase, as measured by the residual biological activity.

The theoretical functions are computed on the assumption of random DNase "hits" in a ring structure containing no, one, or two maximally separated blocks to *E. coli* phosphodiesterase action. The functions are, $m$ hits: for no block $[1-e^{-m}]$; for one block, $[(m+e^{-m}-1)/m]$; for two blocks, $[(m+2\ e^{-m/2}-2)/m]$.

against the number of hits produced by the nuclease (calculated, with good agreement, from either the decline in biological activity or in the amount of the $S_1$ component) they are found to fit the function expected if, after opening of the ring, there is a single discontinuity or block to *E. coli* phosphodiesterase action, randomly located with respect to the first nuclease hit. (The hypothesis of random location is supported by the failure to observe a discrete boundary, other than residual $S_1$, after completion of the *E. coli* phosphodiesterase action.)

That the action of the *E. coli* phosphodiesterase in these experiments was maximal is supported by the observation that the ratio of per cent hydrolysed to the theoretical amount of hydrolysable material (100% minus the proportion of residual $S_1$) increased with increasing hits per molecule, and by direct demonstration, in the case of the 2·2 hit sample, that dialysis, lyophilization, and re-incubation with *E. coli* phosphodiesterase resulted in no further degradation.

These observations account for the consistent failure in earlier experiments to remove completely the slow-moving components by enzymic action (Table 5).

## 4. Discussion

The enzymic and centrifugal experiments indicate that the infective $\phi$X-DNA chains are chemically and physically indistinguishable from the $S_1$ component, which has a ring structure. The principal evidence for a ring structure is that the first degradation product has an unchanged, or nearly unchanged, molecular weight, and constitutes physically an homogeneous component, while further chain breaks, which occur at a comparable rate to the first, result in random fragmentation.

Other models might be invented to explain the homogeneity of the first degradation product, which involved an exclusive site for the first either enzymic or thermal hit, resulting in a major component, the $S_2$, and a small fragment which has been overlooked. Such an explanation seems unlikely considering the known broad specificity of pancreatic DNase and the lack of secondary structure in DNA at 100°C. Furthermore, in both types of degradation, the rate of occurrence of the second hit is as large as that of the first, suggesting the similarity of both events. It is also pertinent that the results in the last section demonstrate that $S_2$ is physically, but not chemically, homogeneous. After phosphodiesterase action a distribution of sizes is obtained, indicating that the distance between the chain scission and the enzymic block is random. This result again argues against the possibility of an exclusive first-hit site.

The ring structure explains the earlier failure to find end-groups by enzymic methods. The covalent nature of the ring follows from the persistence of the $S_1$ and $S_2$ boundaries under conditions of complete exclusion of hydrogen bond formation. The ring structure of this viral DNA offers interesting possibilities for understanding the molecular basis of cyclic genomes.

It seems significant that the infective ring molecule contains one discontinuity (resistant to *E. coli* phosphodiesterase) along the primary DNA chain. The nature of this discontinuity is at present unknown. It may be an unusual nucleotide, an unusual linkage, a small hairpin-like tightly hydrogen-bonded region, or the ring may be closed by non-nucleotide components. It should be noted, however, that the two bonds which link this discontinuity to the 3′-OH and 5′-OH ends of the DNA chain, are at least as stable as ordinary phosphodiester bonds under a variety of conditions which include heating in neutral solution and at pH 9, treatment with 0·1 N-sodium

hydroxide at 37°C, and with 0·1 N-acetic acid at 30°C. It is tempting to speculate that this special region may play an important part in the initiation of infection and in the replication of the viral DNA (Sinsheimer, Starman, Nagler & Guthrie, 1962).

We thank Dr. J. Vinograd for showing us prior to publication his data on the behavior of $\phi$X DNA in alkaline solution. Dr. E. Carusi assisted on several occasions with the ultracentrifuge studies. This research was supported by United States Public Health Service Grant RG6965. One of us (W. F.) is a Rockefeller Foundation Fellow; "Chargé de Recherches du Fonds National Belge de la Recherche Scientifique".

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