THE MOLECULAR WEIGHTS OF T2 BACTERIOPHAGE DNA AND ITS FIRST AND SECOND BREAKAGE PRODUCTS

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Recent evidence indicates that DNA molecules of uniform size and high molecular weight can be extracted from phage T2.[1, 2] Controlled degradation by stirring yields fragments tentatively identified as halves and quarters of the original molecules.[3] In previous work, these materials were partially characterized by physical methods, but their absolute molecular weights remained unknown.

Our purpose in the work reported here was threefold: to determine the size and number of DNA molecules per phage particle, to check these measurements against the putative half and quarter molecules (and vice versa), and to calibrate sedimentation coefficients for DNA in terms of molecular weight in a high molecular weight range previously unmeasurable. These objectives are pertinent to the organization of the phage "chromosome," which by genetic criteria is a single structure.[4]

We show below that, contrary to earlier indications,[2, 3] the particle of phage T2 contains indeed a single molecule of DNA of molecular weight at least 130 million. Some of our results are supported by those presented in the companion paper by Davison, Freifelder, Hede, and Levinthal.[5]

Our conclusions are reached by employing a method of molecular autoradiography.[6] When phage particles or DNA molecules labeled with P^{32} are embedded in a radiation-sensitive ("nuclear") emulsion, the emitted β-particles produce tracks which, emanating from a point source, give rise to characteristic figures recognizable as "stars." If it is assumed that every β-particle forms a track, counts of the average number of tracks per star measure the number of P^{32} atoms per particle and, depending on the specific radioactivity, the total phosphorus content per particle. Less accurately, the number of labeled particles can be measured from the ratio of stars to added polystyrene latex spheres.

Materials and Methods.—Escherichia coli strain H was grown for 2 hours with aeration at 36°C in 7 ml of a synthetic culture medium,[7] to which was added 0.2 mg/ml of bacteriological peptone and the required amount of radiophosphate (Squibb and Sons, 1,000 c/g). The total phosphorus content of the medium (about 3 μg/ml) was measured colorimetrically and the P^{32} content was determined by comparison with a standard P^{32} solution (calibrated by the National Bureau of Standards). After the bacterial mass had increased 10-fold and the number approached 10^8/ml, the culture was infected with three particles of phage T2H per bacterium. One hour later the culture was lysed by shaking with a few drops of $CHCl_3$ and digested serially for 10 minutes each with small quantities of deoxyribonuclease, ribonuclease, and pancreatin. After this treatment 10^{11}/ml of carrier phage, heavily irradiated with a germicidal lamp, were added and the lysate was filtered through a layer of analytical grade Celite on filter paper in a small Gooch crucible. A sample of the filtrate was diluted 10^6-fold in buffered peptone solution[8] for daily titration of surviving phage particles.

Five ml of the filtrate was mixed with 1.8×10^{13} purified unlabeled phage particles. An aliquot of this mixture was purified by centrifugation for assay of P^{32} and DNA contents, and for the star-size measurements of whole phage particles. The remainder of the mixture (2×10^{12} phage per ml) was shaken twice with phenol and five times with ether to yield 8 ml of DNA solution (about 0.4 mg/ml.)[9] The carrier phage provide a high concentration of DNA which serves to protect both the labeled and unlabeled molecules from breakage during extraction. Also, the

large excess of unlabeled molecules prevents the aggregation of labeled molecules which would distort the autoradiographic results.

Aliquots of the DNA solution were stirred under conditions ("5,000 rpm") expected to produce mainly half molecules, or ("9,000 rpm") to produce mainly quarter molecules.[3] Stirred and unstirred samples were then passed through separate fractionating columns[9] and the resulting fractions were analyzed spectrophotometrically for DNA content and by radioassay for P^{32} content. Fractions were selected for embedding in the nuclear emulsion.

It should be noted that the procedure described avoids purification of radioactive phage particles before extraction of the DNA and relies on the column to effect final purification. This procedure prevents possible radiation damage to phage particles packed in the centrifuge, shortens the time required to get the DNA into the emulsion, and undoubtedly removes some broken or radiation-damaged DNA from the preparations.[9] We observed that the labeled DNA does in fact fragment at an appreciable daily rate, dependent on specific radioactivity, even when stored at effectively infinite dilution. In the experiments reported here, DNA was embedded in the emulsion within 24 hours or less after the time of synthesis. Less than four atoms of P^{32} per molecule of DNA decayed in the interim.

A few modifications of the autoradiographic procedures[6] were introduced mainly to minimize inadvertent mechanical breakage of the DNA. Samples were diluted with carrier DNA solutions (40 to 400 μg/ml) and volumes were measured in wide-mouth pipettes operated by screw-delivery. Solutions were mixed mechanically in tubes rotated at 10 rpm while inclined at 30° to the horizon. Five ml of melted emulsion and 1 ml of DNA (total 400 μg containing 10,000 to 50,000 labeled particles and an equal number of styrene latex spheres) were rotated for 5 minutes while submerged in water at 45°C. In a control test DNA of very high specific radioactivity was mixed with emulsion, and two drops of the mixture (the maximum amount compatible with satisfactory chromatography) were passed through the column. This test revealed that not more than 8% mechanical breakage was caused by the mixing.

Samples of the emulsion mixtures were poured into glass rings attached with paraffin to coated glass slides[6] to form a disk two to three mm thick before drying. After drying, the plates were stored for not more than two weeks and replicate samples were developed at appropriate intervals. They were viewed with an oil immersion objective at a total magnification of 575 ×. The specimens were counted by two observers (I. R. and C. A. T.) and the results were compared, usually with good agreement. Histograms shown in this paper include the results of both observers.

The number of styrene latex spheres[6] added to the emulsion was determined by counting an aqueous suspension in a calibrated Petroff-Hauser bacterial counting chamber. The ratio of spheres to stars counted in the emulsion measured the number of radioactive particles per ml and, together with the P^{32} assay, the number per phage equivalent of radioactive DNA.

Sedimentation constants were measured in an aluminum cell at 35,600 rpm, 10 μg DNA/ml, in 0.72 *M* NaCl, pH 6.7, at 22°C. and are reported without correction.[3]

DNA concentrations were measured spectrophotometrically in terms of the specific extinction (carefully measured for T2 DNA both before and after chromatographic purification) of 0.208 $cm^2/\mu g$ P or 0.0181 $cm^2/\mu g$ DNA. The latter figure, and all molecular weights cited in this paper, refer to the sodium nucleate with an average residue weight of 357 daltons, 8.7% P.

We also measured the specific extinction of phage particles: 0.311 $cm^2/\mu g$ P at 260 mμ. Since 98% or more of the phosphorus in phage particles is in DNA,[2] the absorbancy includes a contribution of 0.208/0.311 = 67% from the DNA and a contribution of 33% mainly due to light scattering. The absorbancy of phage particles was therefore corrected for scattering by subtracting 33% of the observed optical density.

Results.—Our principal results are derived from three independent experiments of the type just described. Their general plan is depicted schematically in Figure 1A.

Characterization of labeled DNA molecules: Our objective in this work is to characterize *unlabeled* DNA molecules. On the other hand, the autoradiographic information is obtained from *labeled* molecules. Therefore, it is important to demonstrate that the two classes of molecules are identical in the properties we seek to measure.

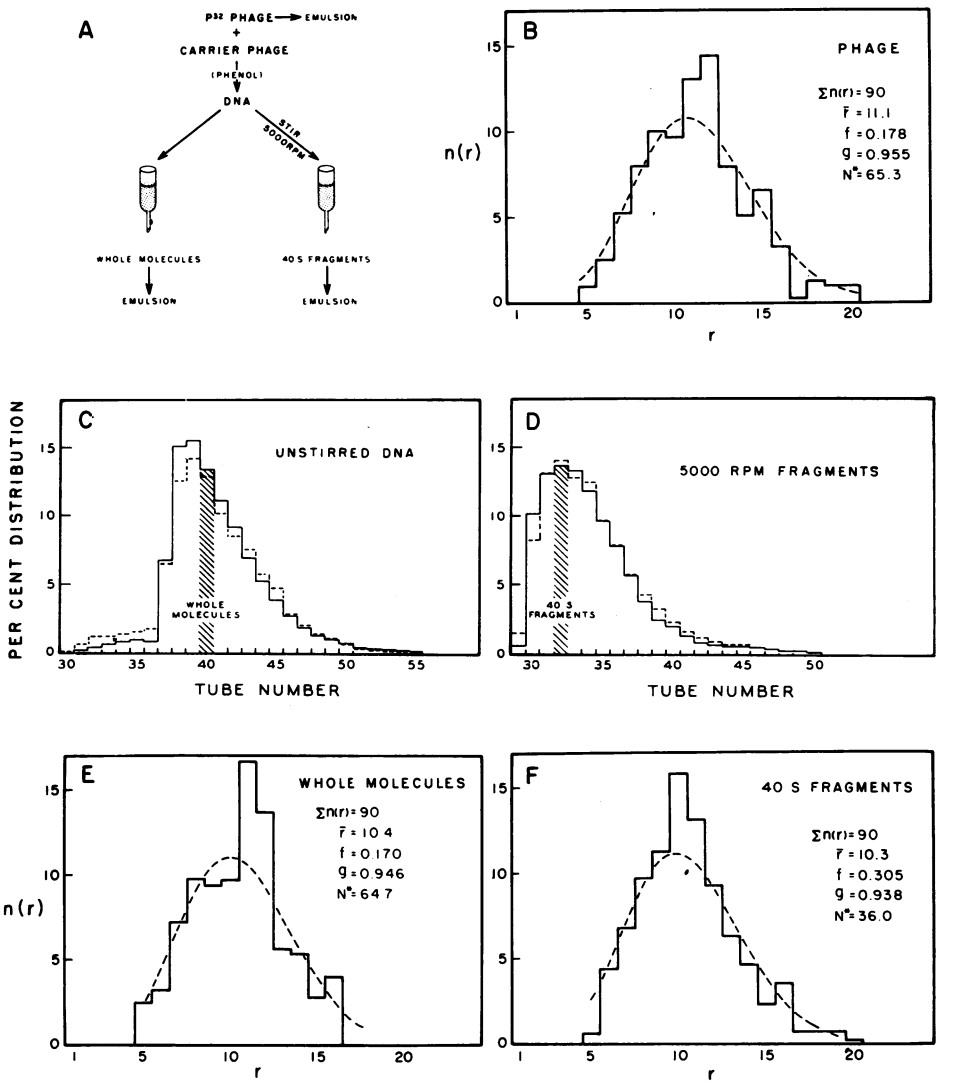

FIG. 1.—Results of Experiment 2.

A, experimental plan. *B*, *E*, and *F*, frequency distributions of star sizes. *C* and *D*, DNA chromatograms. Solid line, optical density; broken line, P^{32}. The shaded fractions were selected for autoradiography.

In Figures 1 and 2 the chromatograms of the mixed DNA preparations are shown. In all cases the elution of the P^{32} and optical density overlaps quite well. This indicates that, of the molecules which pass through the column, the two classes have similar chromatographic properties and similar sensitivity to breakage by stirring.

There is little preferential retention of labeled molecules by the column. This is evidenced by the constancy of the specific activity determinations listed in Table 1. Here one can see that the specific activity of the mixed phage stock or of the extracted DNA is essentially the same as that of the DNA fractions collected from the column.

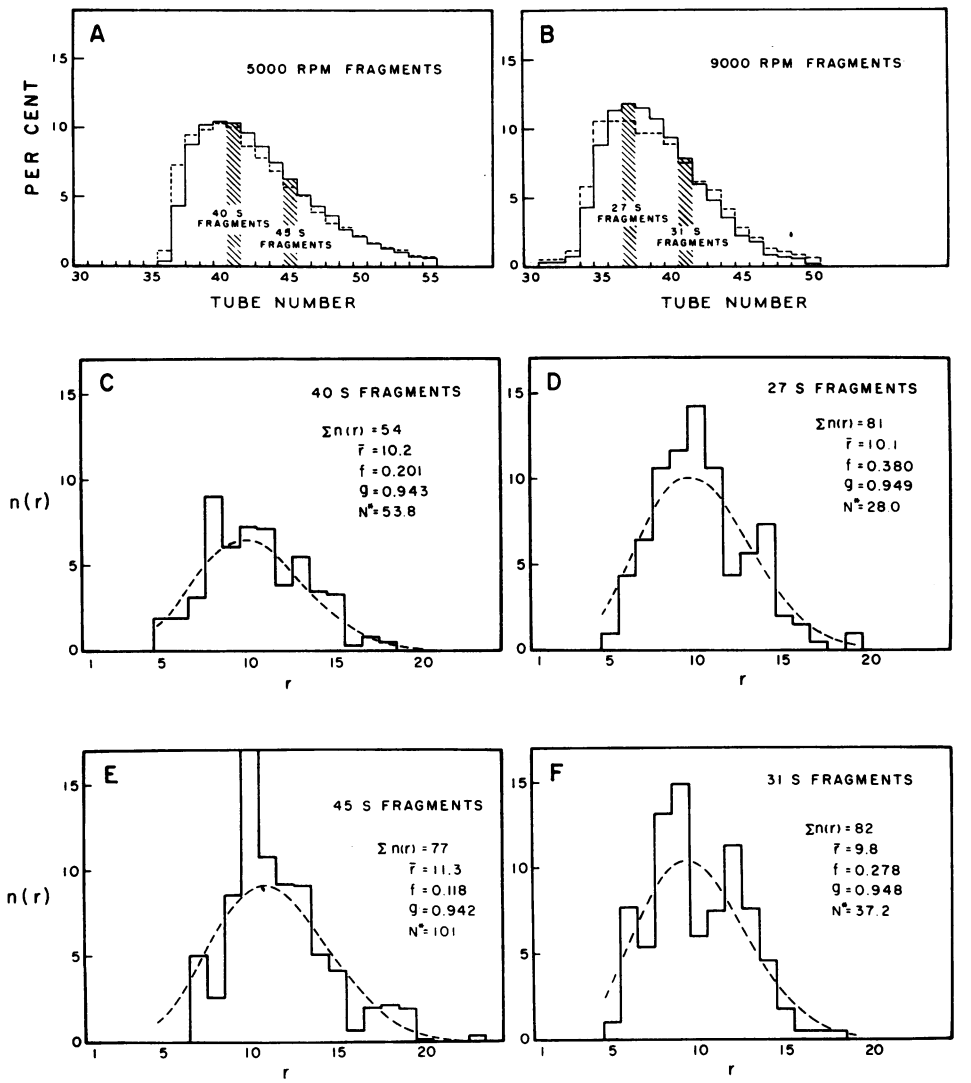

FIG. 2.—Results of Experiment 3. See legend, Fig. 1.

To summarize, labeled and unlabeled DNA respond similarly to stirring and column chromatography.

The first two entries in Table 1 show determinations of the specific radioactivity of the growth medium, the first by direct measurement, the second by employing an empirical relation between the rate of phage inactivation by "suicide" and the specific activity of the growth medium.[10, 11] The two determinations agree. The suicide experiments gave linear plots indicating that more than 90% of the viable phage had been uniformly labeled at the specific activities quoted.

Table 2 lists the sedimentation velocity measurements that were made on pertinent fractions from the column. Because of the similar response of the labeled and unlabeled DNA to stirring and column chromatography it is assumed that their sedimentation constants were also the same.

TABLE 1

SPECIFIC RADIOACTIVITY MEASUREMENTS

	Expt. 1	Expt. 2	Expt. 3
Growth medium (c/g P)	71	49	109
Same, from suicide rate of phage*	70	54	106
Labeled + carrier phage†	13,000	3,800	1,000
Extracted DNA‡	9,200	3,800	950
Unbroken DNA from column	13,000	3,800	...
5000 rpm, 45 S fragments	...	...	740
5000 rpm, 40 S fragments	13,000	3,700	790
9000 rpm, 31 S fragments	...	...	830
9000 rpm, 27 S fragments	...	...	750

* Equation (2) of Hershey *et al.*,[10] αN = 43,000.
† Cpm/μg DNA, corrected for 33% scattering contribution to the absorbancy at 260 mμ. The specific radioactivities of DNA are not simply related to those of the growth medium because of the arbitrary admixture of carrier phage.
‡ Cpm/μg DNA. Sample purified before radioassay by Schmidt-Thannhauser fractionation.[7]

TABLE 2

SEDIMENTATION COEFFICIENTS OF DNA FRACTIONS

	Expt. No.	s (Svedbergs)
Whole molecules	1	61.4 (..., 64.0)*
45 S fragments	3	45.3 (43.9, 47.2)
40 S fragments	1	40.7 (38.7, 41.6)
"	2	40.0 (36.8, 43.3)
"	3	40.0 (38.4, 41.9)
31 S fragments	3	31.3 (29.5, 31.7)
27 S fragments	3	27.4 (23.8, 27.7)

* Figures in parentheses are sedimentation coefficients of fractions eluting from the columns imediately before and after, respectively, the fractions used for star counts. They serve as checks.

Autoradiography: The β-ray stars arising from the labeled phage particles and the DNA molecules were counted and grouped into size classes according to the number of rays per star. Some of the resulting frequency distributions are shown in Figures 1 and 2. The dotted curve in each of the histograms represents a Poisson distribution having the same mean and size as the star population. As one can see, all of the star populations follow a distribution reasonably close to the Poisson distribution. In each star histogram the following numbers are quoted: $\Sigma n(r)$, the number of stars counted in this collection; $\bar{r}$, the mean number of rays per star; f, the fraction of P^{32} atoms that decayed in the emulsion; g, the fraction of P^{32} atoms that remained at the time of embedding (used to correct all N^* values to the time of phage growth); and N^*, the calculated number of P^{32} atoms per star-forming unit found by dividing $\bar{r}$ by fg.

The autoradiographic results are summarized in Table 3. In the first column for each of the three experiments is seen N^*, the calculated number of P^{32} atoms per star-forming unit, and in brackets the total number of stars counted. In some cases these data were collected from plates that had been exposed for different lengths of time. The values of N^* were in good agreement indicating that the efficiency of β-ray detection was the same for plates stored for different lengths of time.[6, 12]

The number of P^{32} atoms per phage, N_0^*, was determined in each experiment. Hence, the ratio N^*/N_0^* in Table 3 indicates the fractional P^{32} content of the DNA molecules as compared to the P^{32} content of the phage particle. This ratio is approximately one for the whole DNA molecule, a fact which indicates that there is only one molecule per phage particle. This ratio is about one-half for the 45 S and

TABLE 3

RELATIVE MOLECULAR WEIGHTS

	Experiment 1			Experiment 2			Experiment 3		
	N^*	N^*/N_0^*	$(s/s_0)^{1.88}$	N^*	N^*/N_0^*	$(s/s_0)^{1.88}$	N^*	N^*/N_0^*	$(s/s_0)^{1.88}$
Phage	92.4 (31)	1.00	...	65.3 (90)	1.00	...	130.1 (91)	1.00	...
Whole molecules	83.4 (178)	0.90	1.00	64.7 (90)	0.99	1.00	...	...	1.00
45 S fragments	...	...	...	...	...	...	94.9 (128)	0.73†	0.54
40 S fragments	39.3 (19)	0.43	0.42	33.1 (196)	0.51	0.43	53.8 (54)	0.41	0.43
31 S fragments	...	...	...	...	...	...	37.2 (82)	0.29	0.27
27 S fragments	...	...	...	...	...	...	28.0 (81)	0.22	0.21

N^* = number of P^{32} atoms per star-forming unit measured autoradiographically, with number of stars counted in parentheses.
N^*/N_0^* = fractional phosphorus content.
$(s/s_0)^{1.88}$ = fractional molecular weight from sedimentation coefficients in Table 2 according to Burgi and Hershey.[3]
† This measurement is presumably in error.

40 S fragments, which represent "half molecules," and about one-quarter for the 31 S and 27 S fragments, which represent "quarter molecules."

Finally, the fractional molecular weight, $(s/s_0)^{1.88}$, calculated from the sedimentation coefficient is listed in the third column. In this expression s_0 is the nominal average (63) of many measurements on unbroken DNA (values range from 60 to 65). The exponent 1.88 is taken from the work of Burgi and Hershey,[3] whose estimates are 1/0.55 for the upper portion and 1/0.51 for the lower portion of the range of s here considered. One may see that the fractional phosphorus content determined by autoradiography corresponds with the fractional molecular weight computed from the sedimentation coefficient.

An independent measure of the number and size of the fragments can be obtained by employing polystyrene latex spheres to measure the number of stars per phage-equivalent of radioactivity. For experiments 1 and 2, respectively, this ratio was 1.52 and 1.15 for intact phage, and 1.14 and 1.36 for unbroken DNA molecules. In experiment 3, the number of stars per phage equivalent of counts ranged from 1.35 for the 45 S fragments to 5.68 for the 27 S fragments. Although these data are qualitatively clear, they lack the precision that was expected. After a consideration of many sources of error, we concluded that we were undercounting the number of polystyrene indicator particles.

Absolute molecular weights: Our conclusion that the T2 phage particle contains a single molecule of DNA reduces the problem of molecular weight measurement to that of determining the amount of DNA per phage particle. From the autoradiographic data of Table 3, five such estimates can be made. The appropriate relation is

$$M = 1.05 \times 10^8 \frac{N^*}{A_0} \qquad (1)$$

where A_0 is the specific radioactivity in c/g, the DNA is assumed to contain 8.7%

P, and all β-emissions are assumed to produce countable tracks in the emulsion. The estimates of N^* (from the counts of phage particles and unbroken DNA molecules) listed in Table 3 together with the specific activities given in Table 1 yield five measures of M ranging from 123 to 140 $\times$ 10^6, with a mean of 133 $\times$ 10^6. The measurements of N^* are evidently repeatable (as is also shown by the measurements of broken DNA) and are subject to a purely statistical error of only a few percent. The measurements do contain a known bias, since it is not certain that all of the emissions produce countable tracks in the emulsion. On this basis 133 million is a minimum estimate.

A maximum molecular weight is obtained by direct measurements of DNA per infective phage particle. A careful analysis of three phage preparations yielded an optical cross section at 260 mμ of 7.3, 7.5, and 7.6 $\times$ 10^{-12} cm^2/plaque-forming particle and 2.3, 2.3, and 2.5 $\times$ 10^{-11} μg P per plaque-forming particle, respectively. These yield an average sodium polynucleotide molecular weight of 162 $\times$ 10^6, which is a maximum since it is unlikely that every phage particle forms a plaque.

We conclude that the sodium polynucleotide molecular weight of T2 DNA lies between 130 $\times$ 10^6 and 160 $\times$ 10^6. The lower limit is consistent with a recent estimate of the weight of the whole phage particle based on sedimentation-diffusion measurement.[13] The phage particle weight was found to be 215 $\times$ 10^6 daltons, which correspond to a DNA molecular weight of 130 million if the phosphorus content of the dry phage is 5.2%.[14]

If the molecular weight is taken to be 130 $\times$ 10^6, the relationship between sedimentation coefficient and molecular weight is that shown in Figure 3. The best straight line corresponds to the equation

$$s = 0.00244\ M^{0.543} \qquad (2)$$

The coefficient of this equation has a standard error of less than 10%, while the error in the exponent is less than 1%. This relation disagrees with a commonly employed extrapolation from the low molecular weight region.[15] The exponent 0.543 agrees with that arrived at by stepwise degradation by stirring.[3]

Systematic errors: The errors in the autoradiographic technique have been discussed previously[6] and only a few comments will be made here.

First, we recall that the possibility that our results are biased by molecular aggregation is ruled out by the method of measurement, in which a small minority of radioactive molecules is examined in the presence of a large excess of carrier DNA.

It is possible that there is some difficulty in the enumeration and classification of the stars shown in the histograms in Figures 1 and 2. Stars with only a few (five to six) rays can be classified accurately, but they are not always seen. Stars with 18 to 25 rays are easy to find, but there is probably some tendency to undercount the number of rays. Thus there is a tendency to overestimate the mean number when it is small, and to underestimate it when large. When the mean lies between 9 and 13 rays per star, there may be a tendency to see a narrower size distribution than actually exists.

To counteract this tendency, plates that had been exposed for various periods were examined. After long exposures, it proved feasible to count rays per star for small stars and at the same time score the total number of stars. The observed

frequency of small stars was in reasonable agreement with the Poisson distribution based on counts made at earlier times.

In the extreme case, the tendencies discussed above could be expected to generate a spurious Poisson distribution when the distribution was in fact very broad. Three experiments with phage T4 performed shortly before those reported above are pertinent in this connection. In the T4 experiments, radioactive and carrier phage particles were mixed and purified by differential centrifugation, and DNA was extracted from them either by osmotic shock or by the phenol method. In each case, the DNA preparations yielded many stars equivalent in size to those produced by phage particles, but also yielded smaller stars. Figure 4 illustrates the broad,

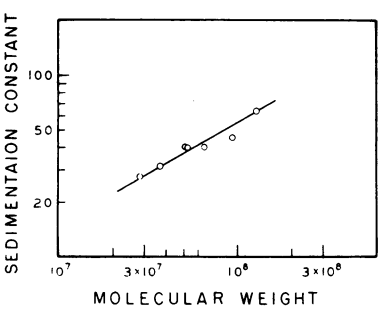

FIG. 3.—The relation between autoradiographically determined molecular weight and sedimentation coefficient for T2 DNA and its breakage products. The straight line is given by equation (2).

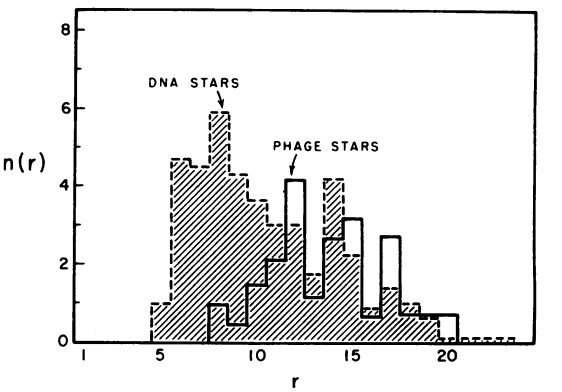

FIG. 4.—Frequency distribution of star sizes for T4 phage and DNA. The DNA was apparently partly broken during preparation.

often bimodal, distributions observed. These results certainly do not mean that the DNA of T4 differs from that of T2. Both have the same sedimentation coefficient and are broken at the same critical speed of stirring. The two phages show the same DNA content per infective particle, and are closely related by all biological criteria. For reasons that have not been determined, the T4 DNA preparations must have been partly broken before embedding. Since each break produces two fragments, very little breakage is sufficient to distort the histograms.

For present purposes, the results of these unsuccessful experiments are valuable in showing that one can detect by autoradiography heterogeneous DNA populations when they exist.

Another source of error anticipated in these experiments failed to materialize. Since DNA solutions are highly viscous, the molecules must be greatly extended in solution. In the extreme cases, such molecules might fail to produce countable stars or might produce spuriously small ones. In point of fact, some two or three per cent of the countable stars appeared to have diffuse centers as much as 10 to 20 microns across. The remainder of the DNA stars, like all of those produced by phage particles, emanated from a single point. Evidently most of the molecules are crumpled in the emulsion so as to lie within volumes not greatly exceeding one micron in diameter. This is shown most convincingly by the similarity of the star-size distributions of T2 phage particles and whole DNA molecules in the present work (Figs. 1*B* and *E*).

To summarize, after considerable experience we have not found any significant source of error in the autoradiographic method that might tend to bias the results or alter the conclusions presented in this paper.

The stability of the DNA molecule: We refer to the DNA structures isolated from phage T2 as "molecules" because of their physical and functional uniformity. The possibility remains that nonphosphodiester linkages exist uniting the polynucleotide chains. It is appropriate to summarize some results which indicate that the molecule is a stable structure.

Passing DNA through the column does not alter its sedimentation coefficient or the band width seen in a CsCl density gradient. Banding in CsCl does not alter the chromatographic behavior. Heating whole molecules to 70°C for 10 minutes in 0.1 *M* NaCl, 0.032 *M* PO_4, pH 7 does not alter the chromatographic behavior of T2 DNA nor diminish its intrinsic viscosity. The star population from T4 DNA (similar to that in Fig. 4) is not altered by heating under the conditions stated above. The intrinsic viscosity of T4 DNA is not altered by prolonged exposure to protease.[2] Study of the kinetics of breakage[3] indicate that the molecules are not characterized by a few weak bonds. The presumed separation of polynucleotide chains from the helical structure is accompanied by only a two-fold decrease in apparent molecular weight.[16] Thus, if unknown linkages exist, they must be at least as stable as the polynucleotide bonds.

Discussion.—Our first conclusion, that the particle of T2 contains a single molecule of DNA, seems quite unambiguous and has been reached independently by Davison *et al.*[5] Previous autoradiographic studies[6, 12] leading to a different conclusion were undoubtedly influenced by the then unknown susceptibility of the molecules to mechanical breakage,[17] though certain details of the early results remain a mystery.

The finding that there exists a single DNA molecule in T2 bacteriophage is consistent with the fact that the known genes in T2 form a single linkage group[4] and with the idea that the "chromosome" is a molecular structure. Phage T2 belongs to a class of phages having the largest known particle size. One of the smallest, phage ϕX-174, also contains a single DNA molecule, in this case single-stranded, with a molecular weight of only 1.7×10^6 (see ref. 18). One may anticipate that other phages will be found to contain a single molecule of nucleic acid. If so, it follows that different viruses will provide a continuous series of unique DNAs covering a wide range of molecular weights.

Our second conclusion, that molecules of T2 DNA break successively into halves and quarters at critical rates of shear, suggests that the molecules are linear. Recent evidence that the genetic map of phage T4 is circular[19] might suggest that the DNA molecule is ring-shaped. Ring-shaped molecules would be expected to break under shear into fragments of $^1/_3$ to $^1/_5$ the original length of the molecule.[3] Since T2 and T4 DNAs are very similar, it is unlikely that either molecule has a circular shape, at least after phenol extraction.

Our third conclusion, that the molecular weight of T2 DNA lies between 130×10^6 and 160×10^6, likewise seems unambiguous. For the molecular weight to be lower than these limits, it would be necessary for the observers to count β-disintegrations that did not occur, which is unlikely, if not impossible. Moreover, the molecular weight measured autoradiographically is supported by estimates based

on the mass and composition of the phage particles.[13]

The physical-chemical methods of molecular weight determination of DNA do not provide much assistance here; indeed it was one of the objectives of this work to relate molecular weight and sedimentation coefficient. In short, the molecular weights calculated from sedimentation and intrinsic viscosity are about a factor of two too low to agree with the autoradiography. Molecular weights calculated from band widths in CsCl are also too low.[2]

Summary.—The bacteriophage T2 contains a single molecule of DNA which accounts for virtually all of the phosphorus of the virus particle.

The first and second breakage products produced by controlled stirring are, on the average, one-half and one-quarter of the total molecular weight.

The sodium salt of T2 DNA has a molecular weight of at least 130×10^6 and less than 160×10^6.

An empirical relation between molecular weight and sedimentation coefficient over a five-fold range downward from 130×10^6 is proposed.

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