Studies on Transformations of *Hemophilus influenzae*

II. The molecular weight of transforming DNA by sedimentation and diffusion measurements

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ABSTRACT The sedimentation and diffusion coefficients have been determined for *Hemophilus influenzae* transforming activity and DNA using P^{32}-labeled DNA. The methods employed the Spinco fixed boundary separation cell for measurements of the sedimentation coefficient and the Northrop-Anson diffusion cell to determine the diffusion coefficient. There was a very close correlation between the amount of DNA and transforming activity sedimented or diffused. The sedimentation coefficient ($s_{20°}$), for both biological activity and DNA was 27 and the diffusion coefficient ($D_{20°}$) 1×10^{-8} cm²/sec. The molecular weight calculated from these coefficients gave a value of 16 million. There was no difference in the sedimentation coefficients for the two unlinked markers, streptomycin and erythromycin resistance, and the diffusion coefficients for single markers or the linked markers, streptomycin and cathomycin, were the same.

INTRODUCTION

The elucidation of the function and structure of DNA has been considerably enhanced in recent years by the study of bacterial transformations which involve the interaction between competent bacterial cells and molecules of DNA. Work in a number of laboratories has shown that a direct relationship exists between the amount of DNA removed from solution and the number of transformations produced. Neither the size nor the chemical composition of these molecules, other than the fact that they carry specific nucleotides, is known. A variety of methods has been applied to the determination of molecular weight of DNA with considerable variation in the values obtained. Many justifiable criticisms may be applied to some of these studies which would suggest that either the DNA used was degraded material or that the

method of measuring molecular weight left something to be desired. In the study to be discussed below, we have eliminated many of the possible criticisms based on the use of inactive material by making use of biologically active DNA. In addition, we have attempted to eliminate those sources of error which have been associated with the asymmetry of DNA molecules, and interactions in solutions by measuring the sedimentation and diffusion coefficients of very dilute solutions of transforming DNA and from them calculating the molecular weight. Certain variables associated with the measurement of the sedimentation and diffusion coefficients were therefore cancelled out. For the measurement of the diffusion coefficient of DNA transforming activity, it was necessary because of the small diffusion coefficient of DNA to turn to the use of the Northrop-Anson porous disc method (1–3). This method has proved to be reliable for proteins of molecular weight 60,000, but the molecular weights of DNA are considerably greater and the asymmetry of the molecules is relatively enormous. The major problem with regard to the measurement of diffusion coefficients by means of the disc method was the fact that the pore diameter of the disc itself might play a role in affecting the diffusion coefficient of the material. If, however, the pores of the diffusion disc are large relative to the size of the molecules, there appears to be no theoretical reason for doubting the values. The average diameter of the pore of the cells used (Corning, medium) is described by the manufacturer as 10 to 15 microns, which is of the order of five to ten times the random length of the DNA molecules in solution assuming the molecular weight of 16 million which was obtained by use of this method. Diffusion cells made of discs of coarse porosity failed to yield uniform cell constants even with a low molecular weight protein. With this limitation on the diffusion coefficient of transforming DNA the evidence for the molecular weight of 16 million for *Hemophilus influenzae* DNA is presented below. A preliminary account of some of these results has been presented previously (4).

MATERIALS AND METHODS

Sedimentation Procedure The sedimentation coefficients of *Hemophilus influenzae* DNA and transforming activity were determined by the use of the Spinco model E fixed boundary separation cell. The procedure used for the determination of the sedimentation coefficient was that of Sher and Mallette (5). In calculating the time of sedimentation it was necessary to apply a correction to the normal running speed for the time during which the rotor was accelerating and decelerating. During the run the speed was plotted as a function of time. For the acceleration and deceleration, which were almost linear with time, a straight line was extrapolated to the time axis. The total time during acceleration and deceleration divided by three, added to the time at running speed gave the total running time.

Description of Diffusion Apparatus The diffusion apparatus was maintained at

3.98°C ± 0.005° in a 50 liter water bath placed in a refrigerated room at about 5°C. Cooling of the bath was obtained by means of a continuous flow of water through copper tubing at 0°C which was in turn balanced by a small heating element which acted intermittently. The bath was vigorously stirred and the temperature maintained by means of a mercury thermoregulator. The temperature was determined with a Beckman thermometer calibrated against a Bureau of Standards thermometer. The diffusion cells and the shells which hold them were similar to those previously described (1–3). The cell constants were obtained with N/1 HCl and confirmed with 0.5 per cent pepsin.

The DNA used in both the sedimentation and diffusion measurements was dissolved in 0.3 N NaCl + 0.014 M sodium citrate at pH 7.0. This solvent was used also for measurement of standard transformation curves.

Measurement of Biological Activity The transforming DNA preparations used were prepared according to the method presented previously for preparation of purified DNA (6). The measurements for biological activity were performed by two methods, both of which gave the same results. The first method involved use of the agar layer technique, in which expression was permitted to occur for a period of 2 hours on the plate and the culture then overlaid with the selective agent. In the second method, expression was permitted to take place in the transformation tube and a pour-plate procedure was used, in which the agar contained the selective agents. In determining the sedimentation or diffusion coefficient for biological activities, the experimental material was always compared to standard dilutions of the material used for determination of the particular physical coefficient. The standard curve involved use of three tenfold dilutions of the original material in the linear portion of the transformation-concentration curve and led to variances of less than 10 per cent of the number of colonies obtained. The experimental material was usually run in duplicate and often with more than one dilution of the material, and the average error was of the order of 10 to 15 per cent.

Preparation and Chemical and Ultraviolet Determinations of P^{32}-Labeled DNA The preparation of P^{32}-labeled transforming DNA involved the growth of the stock of donor cells in Levinthal broth depleted of phosphorus by passage through a Dowex-1 column. The concentration of phosphorus left after passage through this column was approximately 13 μg/ml. Seven millicuries of P^{32} were added to 400 ml of phosphate-depleted broth and *Hemophilus* cells were added to give a concentration of approximately 10^6/ml. The cells were grown overnight, harvested, and the DNA prepared according to the method previously outlined.

The determination of the DNA content of the preparation was made by measurement of the phosphorus content of the preparation, the diphenylamine reaction by the Burton method (7) using a Worthington thymus DNA preparation as a standard, and by measuring the amount of P^{32} material released after treatment with DNAase, RNAase, or trypsin. The acid-soluble P^{32} released after treatment with each enzyme represented the particular fraction of that material in the DNA preparation. In the three DNA preparations used for determination of sedimentation and diffusion coefficients, the amount of P^{32} was correlated very closely with the amount of DNA,

and represented greater than 99 per cent of the P^{32}. P^{32} measurements were made with a Geiger-Muller end-window counter with a counting efficiency of 20 per cent. For some determinations an adapter was used which permitted an efficiency of counting of 35 per cent. In addition, the 260 mμ absorption in the Beckman spectrophotometer was also determined as well as the ratio of 260 to 230 as a measure of the purity of the DNA preparation.

TABLE I

SEDIMENTATION COEFFICIENTS FOR DNA AND TRANSFORMING FACTOR (T.F.)

Run	Concentration		Speed	Time	Fraction sedimented			s_{20}	
					Theoretical for $s_{20} = 27$	Observed			
	DNA μg/ml	T.F.				P^{32}	T.F.	P^{32}	T.F.
			RPM	*min.*					
1	10	S	39,050	35.3	1.0	0.84	0.89	21.0	22.4
2	1	S	36,950	38.7	1.0	0.82	0.87	20.3	21.8
3	0.1	S	37,300	33.7	0.93	0.80	0.84	23.0	24.2
4	1	S	33,200	23.8	0.53	0.55	0.62	28.0	31.9
5	1	S	32,200	40.4	0.83	0.85	0.91	27.6	29.8
6	1	S	33,130	53.3	1.0	0.90	0.94	23.3	24.6
7	10	S	29,200	22.7	0.4	0.43	0.39	29.6	26.6
8	10	S	33,100	31.3	0.715	0.74	0.78	27.5	29.3
9	5	ES	33,300	33.3	0.91	0.76	0.77	23.8	24.1
10	5	ES	29,300	22.1	0.40	0.44	0.46	30.6	31.6
11	5	ES	55,800	50	1.0	0.97	0.986	9.4	9.6
12	10	S	55,100	59.7	1.0	0.90	0.94	6.6	6.8

EXPERIMENTAL RESULTS

Sedimentation of DNA and Its Transforming Activity

The sedimentation coefficient of P^{32}-labeled transforming factor has been determined for both its DNA content and biological activity. The DNA was uniformly distributed in a Spinco separation cell and sedimented for various periods of time and various speeds, and the P^{32} and biological activities of the material remaining in the top section of the separation cell were then compared to an aliquot of the material prior to sedimenting. The results are shown in Table I. From an analysis of the data given, it was concluded that the sedimentation coefficient of the DNA was the same as the sedimentation coefficient of the transforming activity, although there seemed to be a small (about 4 per cent) fraction of DNA which was not sedimented as rapidly as transforming activity. The sedimentation coefficient of P^{32} differed from the sedimentation coefficients of transforming activity by -2 Svedberg units, which corresponds to a difference of about 4 per cent of the amount of DNA sedimented. Since the analysis of the P^{32}-labeled DNA showed that less than 1

per cent of the material was acid-soluble P^{32} or non-DNA material, it would appear to be likely that the 4 per cent was really DNA.

In addition, it was found that on continued centrifugation some of the P^{32} and transforming activity still remained in the upper section of the separation cell. This activity could be a reflection of the heterogeneity of the DNA. These results are shown in runs 11 and 12 (Table I) and suggest that certainly not more than 10 per cent of the material could be due to such low sedimenting material. However, an additional cycle of centrifugation of the material in the upper portion of the separation cell showed it to have the same sedimentation coefficient as the original material. Therefore, one is led to the conclusion that the material left in the top fraction of the cell was due to malfunction of the separation cell. Except for the centrifugation for long periods at speeds of 50,000 RPM or more, (runs 11 and 12), the sedimentation coefficients are consistent when 40 to 90 per cent of the DNA was removed from the top of the cell. The cause of the malfunction of the cell is not known; however, under some conditions of removal of samples from the top of separation cell, as much as 5 to 10 per cent mixing of the material from the bottom portion of the cell may occur. This fact could explain why there is a greater discrepancy in the s_{20} values obtained after prolonged centrifugation than when shorter periods of time are used and less material sedimented. Although the number of runs is small, there appeared to be no major differences in the sedimentation coefficients obtained for different concentrations of DNA from 0.1 μg/ml to 10 μg/ml and for the two different DNA preparations used. In addition, it was found that for the preparation marked with erythromycin resistance and streptomycin resistance (unlinked markers), there was no difference in the sedimentation coefficients of these two markers. The amount of P^{32} and transforming activity lost from the upper section of the Spinco separation cell was quantitatively recovered from the lower cell as determined by P^{32} measurements or biological activity.

Diffusion Coefficients

The diffusion coefficient of transforming DNA was determined for three different diffusion cells and two preparations of DNA. The diffusion coefficient for biological activity alone was determined in diffusion cells 1 and 3 for the DNA preparation ES used in sedimentation runs 9, 10, and 11 shown in Table I. To measure the diffusion coefficient for both biological activity and P^{32}, the experiment with cell 3 was terminated and the P^{32}-labeled transforming activity was introduced into cells 2 and 3.

The procedure used in carrying out the diffusion measurements was to introduce 20 ml samples of solvent into the shell supporting the diffusion cell so that the bottom of the diffusion disc was covered with solvent. At various

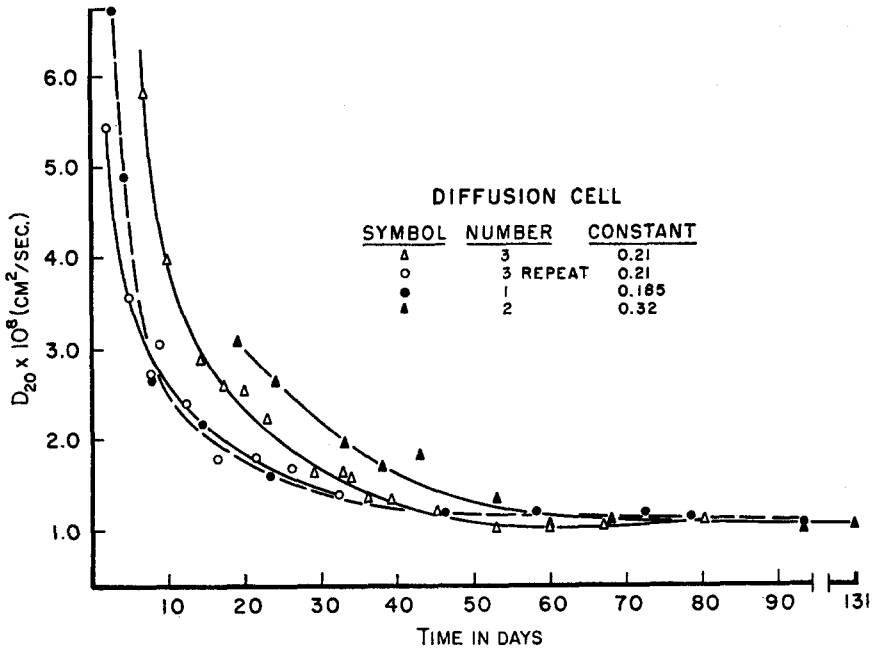

FIGURE 1. Equilibration time of transforming DNA in three Northrop-Anson diffusion cells.

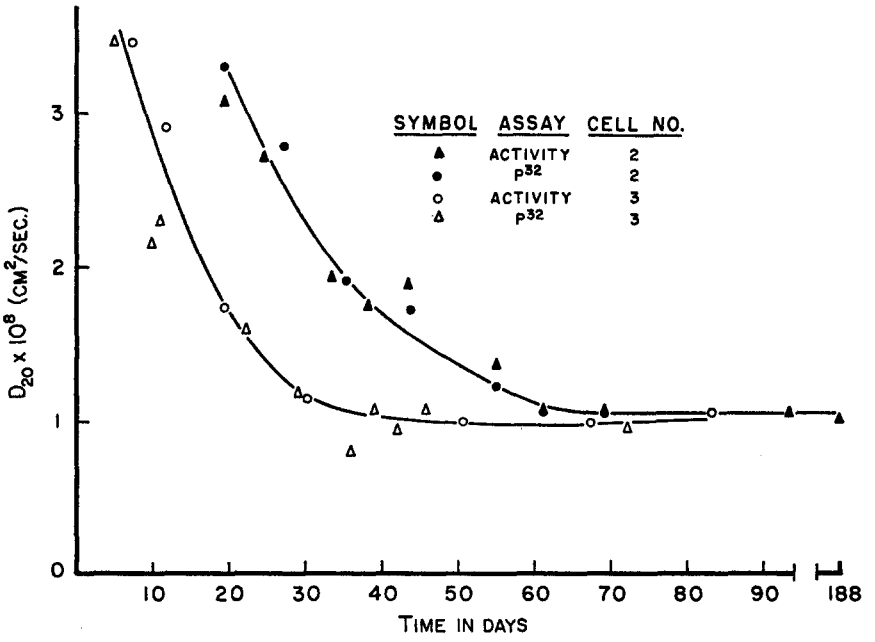

FIGURE 2. Diffusion coefficient of transforming DNA calculated from P^{32} and transforming activities.

periods of time the solvent containing the diffusate was removed, and measurements of P^{32} and biological activity were made, along with known dilutions of the starting material introduced into the diffusion cell. The results are given in Figs. 1 and 2, and show that the diffusion coefficients calculated from P^{32} activity and transforming activity were the same for three cells having different apparatus constants. Although equilibrium conditions were not tested in the first run with cell 3, the shape of this curve and the time necessary to approach equilibrium suggest that the method was reproducible.

In addition to the numerical value of the diffusion coefficient, it was of interest to know whether genetic markers differed in their relative rates of

TABLE II

TIME TO REACH DIFFUSION EQUILIBRIUM FOR TWO TRANSFORMING FACTORS

Sample No.	Time from start of diffusion experiment	Streptomycin resistance $Dt\ 4° \times 10^9$ cm²/sec.	Erythromycin resistance $Dt\ 4° \times 10^9$ cm²/sec.
2	3 hrs.	34	
3	6 hrs.	24	
4	20 hrs.	23	22
8	4 days	17	16
12	8 days	10	12
16	12 days	8.9	9.6
20	21 days	7.4	8.9
24	35 days	8.6	7.4
28	46 days	6.2	6.2
32	55 days	6.1	5.8
36	72 days	6.1	5.8
38	78 days	5.8	5.8
40	93 days	5.6	4.9
42	113 days	5.5	5.1

diffusion. These results are shown in Tables II and III. The diffusion coefficients of the unlinked markers, streptomycin and erythromycin, were found to be the same, and it was also clear that the linked transforming factors, streptomycin-cathomycin, which were present in cell 2 and the second run of cell 3, gave the same diffusion coefficient for the individual transformations for cathomycin and streptomycin and for the linked transformation, S-C. In order to show that the material which had diffused out of the diffusion cells had not been altered in the process, the sedimentation coefficient of this material was determined. The results are given in Table IV and show that the sedimentation coefficients of two diffusion samples were not significantly different from the material first introduced into the diffusion cell. At the termination of the diffusion runs, the amount of transforming activity remaining in the diffusion cells was determined for cells 1 and 3 (first run). The

DNA in cell 2 and the second run of cell 3 was lost after 180 days due to contamination. The actual amount of transforming activity diffused out in this time was about 8 per cent for cell 3 and about 15 per cent for cell 1. The

TABLE III

COMPARISON OF AMOUNTS AND RATIOS OF TRANSFORMING ACTIVITY DIFFUSED OUT FOR S, C, AND SC MARKERS

	Time from start of diffusion experiment	No. of transformations $\times 10^{-3}$			Ratios	
		S	C	SC	S/SC	C/SC
	days					
Cell 3	37	2.0	4.6	0.66	3.0	7.0
	43	1.4	3.5	0.60	2.3	5.8
	54	3.7	9.2	1.3	2.9	7.1
	95	3.6	8.7	1.2	3.0	7.2
	131	6.7	16	2.1	3.2	7.6
	188	1.1	1.9	0.32	3.3	5.9
Cell 2	3				3.0	6.7
	7				3.1	6.5
	17				3.2	7.7
	91				3.2	6.9
	132				3.5	7.1

TABLE IV

SEDIMENTATION COEFFICIENTS OF DIFFUSION SAMPLES TAKEN AT 5 AND 20 DAYS

Sample day	Time	Speed	Fraction of T.F. in top layer	$s_{20°}$
	min.	*RPM*		
5	22.8	29,330	0.60	25
5	32.9	33,350	0.28	25
20	25.6	29,150	0.50	27.5
20	34.0	33,290	0.26	25.4

Samples from a diffusion cell were sedimented for the times and speeds indicated and assayed for streptomycin T.F. remaining in the top section of the Spinco separation cell.

value for the amount of transforming activity remaining in cell 3 was 100 per cent ± 10 per cent, and for cell 1, 90 per cent ± 10 per cent. These data suggested that the DNA was not adsorbed to the glass in the diffusion cell and could freely pass the pores of the diffusion disc. A direct test as to whether or not the diffusion discs would hold back DNA was made by testing the amount of transforming activity which would pass the disc. No transforming

activity was lost from either dilute solutions of 1 and 0.001 μg/ml or solutions of 5 μg/ml.[1]

DISCUSSION

The sedimentation coefficient, $s_{20^\circ} = 27 \pm 3$, and diffusion coefficient, $D_{20^\circ} = 1.0 \times 10^{-8} \pm 0.05$, when introduced into the equation

$$\text{M.W.} = \frac{R \cdot T \cdot s_{20}}{D_{20}(1 - \bar{V}\rho_{20})}$$

where R is the gas constant, T the absolute temperature, s_{20} and D_{20} the sedimentation and diffusion coefficients, $\bar{V}$ the partial specific volume, and ρ_{20} the density, result in a molecular weight of 16,000,000. For the partial specific volume of *H. influenzae* DNA, a value of 0.59 has been used. This figure was obtained by relating the guanine + cytosine content as determined by Zamenhof (12) to the density (13). In a previous report (4), a molecular weight of 14,500,000 was calculated by using a value of 0.55 obtained from the literature (17) as the partial specific volume of DNA. It should be stressed that the value of 16,000,000 obtained for the molecular weight of *H. influenzae* DNA by the sedimentation and diffusion methods used above is an average value and says nothing about the homogeneity or dispersity of the material. The accuracy of the value is estimated to be of the order of 10 per cent. From the molecular weight value the number of transforming DNA molecules in a solution containing a given amount of DNA may be calculated. What one would like to know is, how reliable this value is, and the degree of homogeneity or dispersity of the DNA solution, whose molecular weight has been determined. Neither of these questions can be adequately answered at the present time, for more reliable methods of measurement of the molecular weight of DNA are not readily available. It is time to determine the molecular weight of a given transforming DNA by a variety of methods, including light scattering, density gradient sedimentation, viscosity measurements, etc. Butler and Shooter (9) tested the sedimentation coefficient of the DNA used in our runs 4, 5, and 6, with ultraviolet optics and found a value close to 27. This same value was also obtained by Driskill (8) using ultraviolet optics on her own preparation. There are no other reported values for the diffusion coefficient of transforming DNA.

Introduction of our sedimentation value into the relationship $s_{20} = 0.063$

[1] Light-scattering measurements (14) and the high viscosity of dilute solutions of DNA show that these molecules are highly asymmetric. This explains the unusually low diffusion constant reported here. Appropriate hydration or viscosity data (15) needed to calculate the asymmetry are not available.

$M^{0.37}$ of Doty *et al.* (16), led to a molecular weight value of 13,200,000. This agreement lends some support to the diffusion figure.

It would be desirable to have an independent measurement of the diffusion of DNA, preferably by a method involving free diffusion. The feasibility of determining the free diffusion coefficient of transforming DNA is presently being explored. Regardless of the absolute value of the diffusion coefficient of transforming DNA, the relative diffusion coefficient for different markers as determined by the diffusion disc method was the same for the markers tested. The fact that the diffusion coefficients for streptomycin, cathomycin, and the double S-C, were the same, suggests that the single transformations for streptomycin or cathomycin could not have resulted from breaking a single molecule of linked streptomycin-cathomycin resulting in molecules of reduced size, since it is extremely unlikely that such a break would increase the sedimentation coefficient of these molecules.

The time required for a porous diffusion disc to come to equilibrium is given by the expression $t = 1.2\ (l')^2/D$ (10); D is the diffusion coefficient and l' is the effective thickness of the porous disc. Lea (11) derived a similar formula, but his coefficient was 0.4 instead of 1.2. For small molecules equilibrium is quickly established; *e.g.*, HCl with a diffusion coefficient of 1.78×10^{-5} cm²/sec. for a 1 molar solution at 4°C required only 30 minutes to establish equilibrium in cell 1 with a cell constant of 0.185. For protein it was noted that the first few samples were to be discarded (3), but in general, for material with diffusion coefficients of the order of 1×10^{-7} cm²/sec., equilibrium was established in approximately 1 day. In the case of DNA the time required to reach equilibrium was much longer. The time required for equilibrium to be established for three different cells with *H. influenzae* DNA was approximately 30, 40, and 60 days for cells with constants 0.185, 0.21, and 0.32.

Considering the average thickness of the porous disc to be 0.2 cm, the values obtained are in reasonably good agreement with the theoretical values.

The authors wish to thank Miss Mary Jane Voll for her technical assistance in carrying out the transformation experiments, and Dr. Herbert J. Rapp for his assistance with the technical details of the ultracentrifuge.

This work was supported in part by a research grant E-1218 from the Public Health Service, and Atomic Energy Commission contract AT (30-1)-1371. Dr. Goodgal's work is supported by a Public Health Service Senior Research Fellowship, SF-218.

Received for publication, October 26, 1960.

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