ON THE DIFFUSION RATES OF BACTERIOPHAGES

by

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Measurements of diffusion constants have contributed much to our understanding of the nature of proteins and other high molecular substances in solution. It might be expected that diffusion studies of viruses would add to our knowledge of these substances, but not much work has been done in this field. As MARKHAM, SMITH, AND LEA[1] have pointed out, three methods have been applied to viruses. First, the techniques employed in the studies of proteins have been applied to several viruses. Since these techniques make use of optical methods for the determination of concentrations at various levels, a large amount of purified virus is required, so much that the method cannot at present be generally used. Second, the method of BOURDILLON[2] has also been tried, but the realization of the ideal conditions necessary for consistent results is apparently difficult. In the third place, the porous disc method of NORTHROP AND ANSON[3] has been used by several investigators to investigate plant viruses and bacteriophage. The method of NORTHROP AND ANSON has the advantage of being applicable to impure preparations in concentrations easily attained.

The porous disc method has been applied to bacteriophages by several workers[4,5,6,7,8] and values of the diffusion constants found have been much higher than would be expected from sedimentation studies. The studies on diffusion of bacteriophages have been most recently discussed by HERSHEY, KIMURA, AND BRONFENBRENNER[8] who have attempted to reconcile the size of T2K bacteriophage particles as seen in electron micrographs (80 × 100 mμ) with the size calculated from diffusion constants (4 mμ). They believed that they had evidence of circulation of fluid through the porous disc and thus explained the high diffusion constants.

The multichambered analytical method of POLSON[9,10] seems especially applicable to the study of diffusion constants of bacteriophages since with its use free diffusion occurs and possible anomalies arising from the presence of a porous disc do not occur. An additional advantage is that the cell need not be standardized with substances whose diffusion constants are known, such as KCl, since the values may be calculated directly. Preliminary results from applying the method to the T3 and T4 bacteriophages have already been published[11].

In the present paper, more extensive results obtained with these bacteriophages are reported. The variation of diffusion constant with concentration described is pointed out.

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APPARATUS

The cell used in these studies has already been described[9, 10]. A battery of six was used which was attached to a base plate provided with levelling screws. The cells may be more conveniently prepared as follows: a 1 cm hole is drilled through the center of a cylindrical piece of stainless steel, 5 cm in diameter and 7 cm long. Six cylindrical holes, 5 mm in diameter, are drilled midway between the center of the cylinder and the periphery to a depth of 5 mm from the other end. The cylinder is then cut into the following sections: (a) a thin section, 5 mm thick, to serve as cover; (b) a 1 cm section; (c) a 2 cm section; and (d) a basal section, 3.5 cm thick. The surfaces of the sections are carefully ground and polished so that in apposition they fit perfectly, forming six cylindrical cavities. They are held together with a center pin provided with a thumb screw. The pin is hollowed out to provide space for a thermometer. The sections of the cell are interchangeable.

A thin layer of silicone grease (DOW-CORNING) is applied to the surfaces of the individual sections to render them watertight when clamped together.

For use the cells are fixed in position on the base plate and alternate cavities filled with the solution to be studied and with suitable solvent. By rotation the solvent may be placed over the solution and after an appropriate time the sections may be isolated by rotating the upper sections to the cut-off position.

CALCULATIONS

Diffusion constants were calculated from the following expression which has been derived elsewhere[10].

$$D = \frac{S^2}{C_o^2 A^2} \cdot \frac{\pi}{t} = \frac{C^2 H^2}{C_o^2} \cdot \frac{\pi}{t} \qquad (1)$$

where D = the diffusion constant of Fick's law, S = the amount of solute which has diffused past the initial boundary between solution and solvent in t sec, C_o = the original concentration of the solution, A = the area of cross section in cm^2, C = the mean concentration of solute in the upper section of the cell at time t, and H = the height of the upper section in cm.

From equation (1) the diffusion constant can be calculated. To convert the diffusion constant at one temperature T_1 into that at another temperature T_2, the following equation was employed:

$$D_{T_2} = D_{T_1} \frac{T_2}{T_1} \cdot \frac{\eta_{T1}}{\eta_{T2}} \qquad (2)$$

where D_{T_1} and η_{T_1} are the diffusion constant and viscosity at the absolute temperature T_1, and D_{T_2} and η_{T_2} those at the absolute temperature T_2 respectively.

The particle sizes were calculated from the diffusion constants using the well-known Einstein equation for a spherical particle.

$$D = \frac{RT}{N} \cdot \frac{1}{6\pi r\eta} \qquad (3)$$

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where R is the gas constant, T is the absolute temperature, N is Avogodro's number, r is the radius of the particle, and η is the viscosity of the medium.

METHODS

Stabilizing the Diffusion Column. As extremely dilute solutions (w/o) of virus were employed, it was necessary to stabilize the liquid column into which the process of diffusion took place, because there was too little density difference between the solution and solvent. For that reason, the virus solution was made up in glucose of 1% concentration. This made it possible to obtain a sharp initial boundary between virus solution and medium. In the resulting diffusion process, the glucose establishes a concentration gradient in the cavities in which the virus then diffuses, undisturbed by convection currents. This addition of glucose had little effect on the diffusion of a protein as will be shown later.

The phage concentrations were determined by the plaque counting method. It was attempted to count 50–300 plaques for each determination. The hemoglobin concentrations were determined with the use of the Beckmann spectrophotometer after conversion of hemoglobin to acid hematin. KCl was determined chemically.

Test of the Method on Substances of Known Diffusion Constants. As a control of the method the diffusion constant of KCl was determined. Unfortunately, a relatively high concentration of salt was necessary in order to obtain sufficient material for analysis. 2.95% KCl was diffused against water and a diffusion constant of $1.18 \cdot 10^{-5}$ cm²/sec at 4° C was calculated. After corrections for temperature and viscosity this becomes $1.02 \cdot 10^{-5}$ cm²/sec at 0° C which is about 12% higher than that reported for KCl by LONGSWORTH[12].

Another control of the method was made with human CO hemoglobin. This protein in a concentration of 4.4% in the presence of 1% glucose and 0.15 M NaCl was diffused against 0.15 M NaCl in the same manner as was used below for phage. The relationship C/C_0 for various times is plotted against $\sqrt{t}$ in Fig. 1. As can be seen, the resulting curve is a straight line passing through the origin. The diffusion constant calculated from this experiment is $4.2 \cdot 10^{-7}$ cm²/sec which when corrected for temperature and viscosity becomes $6.70 \cdot 10^{-7}$ cm²/sec at 20° C. Considering the high protein concentration used in this experiment, the agreement between this value and that determined for human hemoglobin by the Lamm scale method, $6.90 \cdot 10^{-7}$ cm²/sec[13] is satisfactory. The glucose that was used for providing a density gradient in the diffusion column had no apparent effect on the progress of diffusion of a protein apart from the usual viscosity retardation effect.

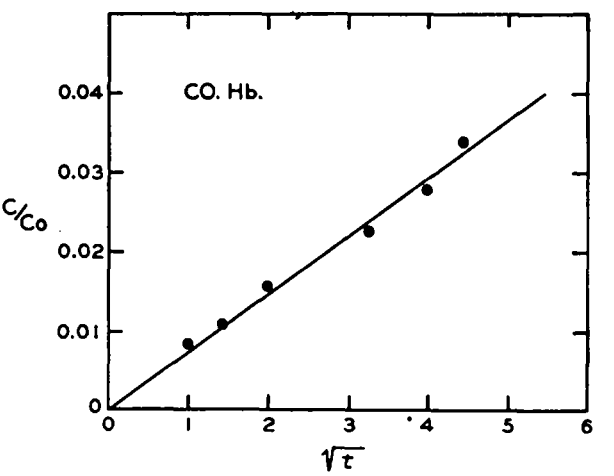

Fig. 1. Diffusion curve of carbon monoxide hemoglobin

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MEASUREMENTS

Unless otherwise stated, the phage solution to be diffused consisted of a dilution of phage in 9 parts of tryptose phosphate broth (Difco) plus 1 part of 10% glucose, and the solvent consisted of tryptose phosphate broth only. This medium has a p_H of

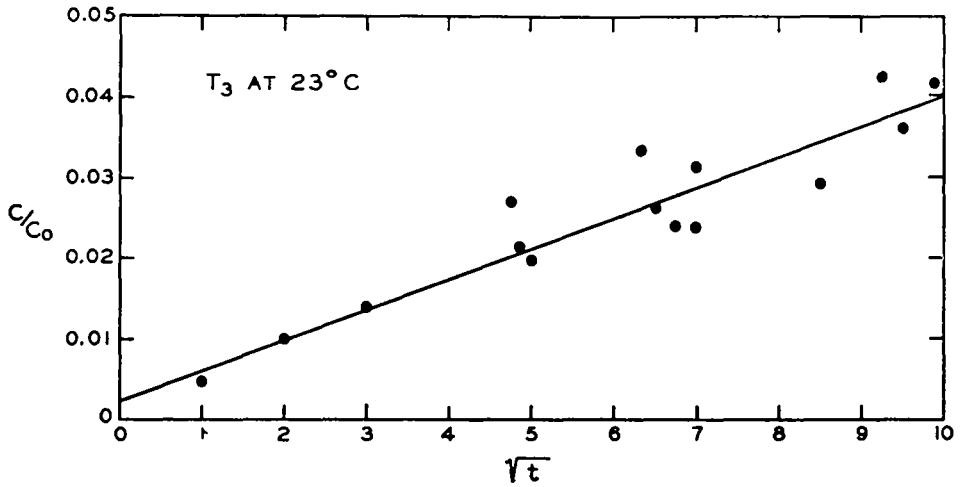

Fig. 2. Diffusion curve of T3 bacteriophage at 23° C

about 7.3 and contains 0.5% NaCl and 0.25% Na_2HPO_4. In Fig. 2 are given results of measurements at 23° C on T_3 phage solutions having phage contents of 10^8 and 10^9 particles/ml. The values are scattered around a straight line from which an average diffusion constant of $1.19 \cdot 10^{-7} cm^2/sec$ at 20° C in water was calculated. From this value a particle diameter of 36.2 mμ for T3 was calculated which is slightly smaller than the value reported by DELBRÜCK from measurements on electron micrographs[14].

The same types of measurement were made on T4 phage having 10^8 and 10^9 particles/ml with the resultant curve in Fig. 3. Again the values determined for C/C_0

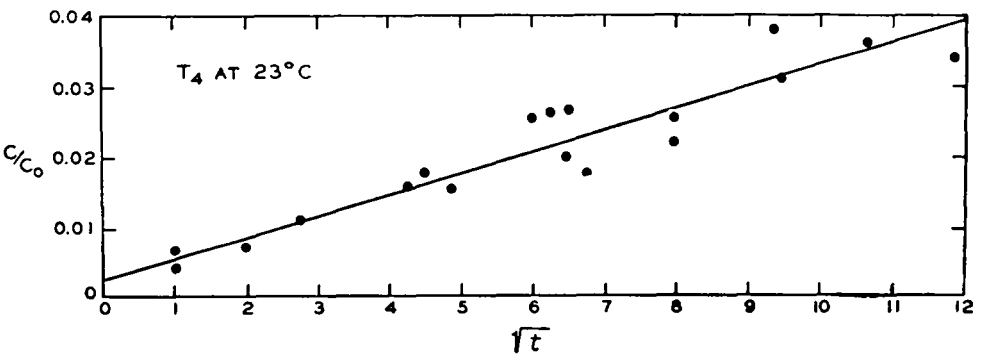

Fig. 3. Diffusion curve of T4 bacteriophage at 23° C

are scattered around a straight line. From the straight line a diffusion constant of $0.8 \cdot 10^{-7} cm^2/sec$ was obtained from which a particle diameter of 55 mμ was calculated. This value is also in fair agreement with that reported by DELBRÜCK[14].

As the determinations were scattered rather much around the mean, it was decided

to repeat them at 4° C at which temperature the most ideal diffusion constants can be determined on account of great stability of the phage and least probability of thermal convection currents.

The results did not come out as would be predicted from temperature and viscosity considerations alone, namely low rates of diffusion were slightly higher at this temperature than at 23° C. This can be seen from Figs 4 and 5.

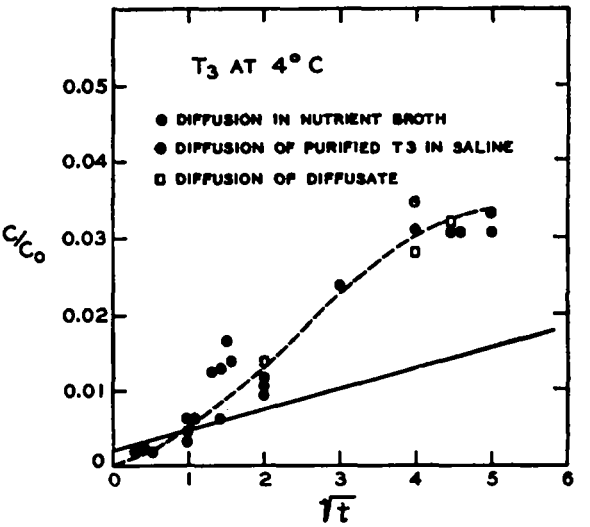

Fig. 4. Diffusion curve of T3 bacteriophage at 4° C. The solid line expresses the value of C/C_0 calculated from the results of Fig. 2, according to Equation (2).

The effect of concentration on diffusion constant was investigated. The results are interesting in that there was a very strong dependency of diffusion rate on the number of phage particles per ml. In Figs 6 and 7 and Table I are given the results at 4° C and at 22° C. At 4° C the diffusion values range from $1.0 \cdot 10^{-7}$ cm²/sec to $5.34 \cdot 10^{-7}$cm²/sec uncorrected for temperature and viscosity for concentrations ranging from $2 \cdot 10^{10}$ particles/ml to $2 \cdot 10^{5}$ particles/ml.

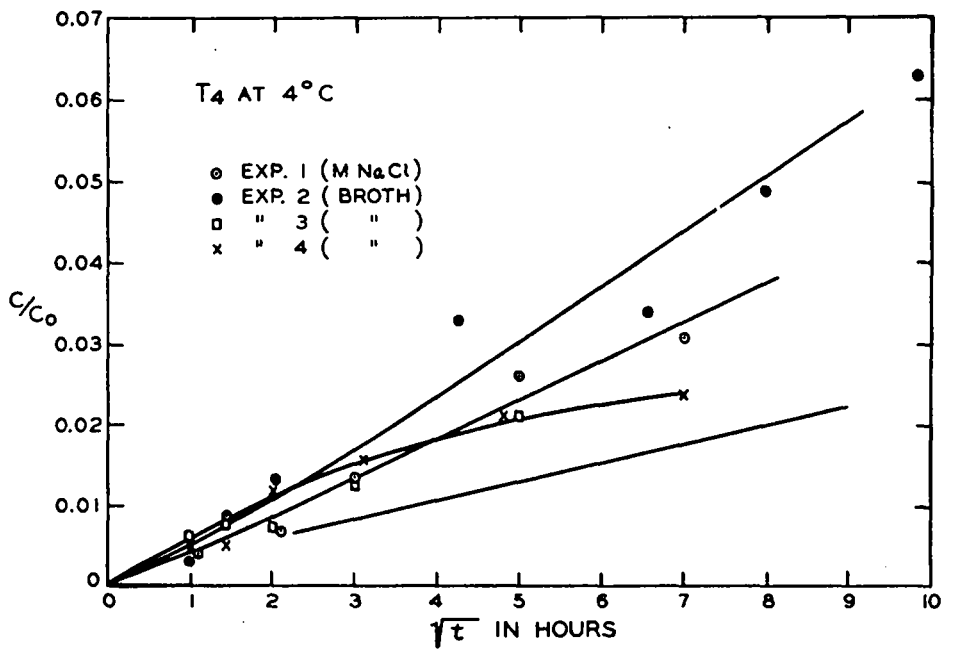

Fig. 5. Diffusion curve of T4 bacteriophage at 4° C. The upper curves refer to different experiments at different original concentrations. The lowest curve expresses the values of C/C_0 calculated from the results of Fig. 3, according to Equation (2).

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At 22° C the variation was greater, here the diffusion constants ranged from $3 \cdot 10^{-7}$cm²/sec to $23.2 \cdot 10^{-7}$cm²/sec for the range of concentration $25 \cdot 10^{8}$ to $25 \cdot 10^{4}$ particles/ml.

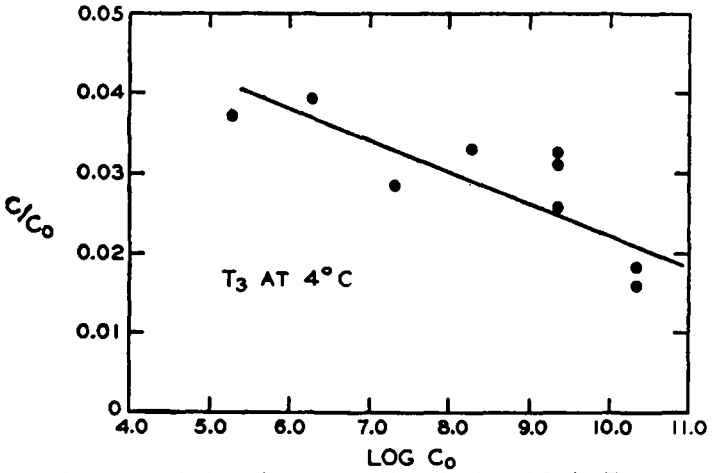

Fig. 6. A plot showing the variation of the ratio C/C_0 with log of C_0 for T_3 bacteriophage at 4° C

Several experiments were conducted without success to find an explanation for this anomalous behaviour of the bacteriophage.

Charge effects. To exclude the possibility that the high diffusion rate was caused

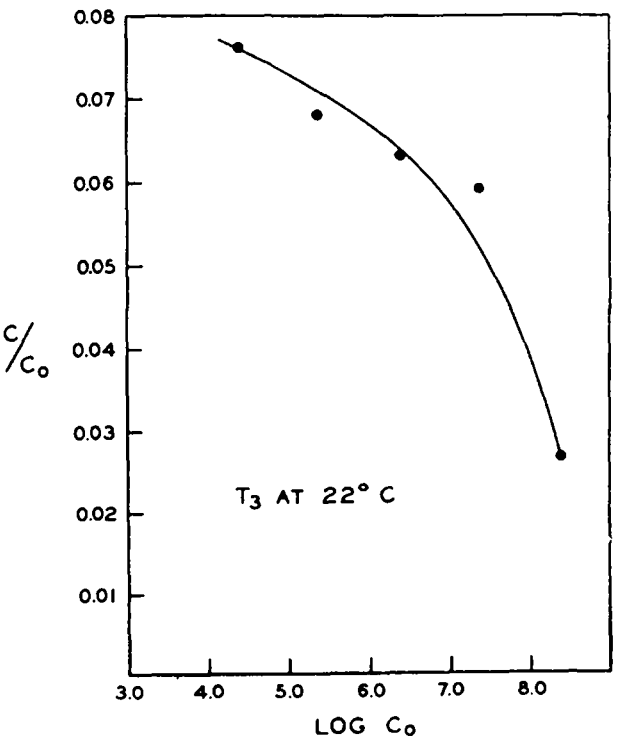

Fig. 7. Variation of the ratio C/C_0 with log of the concentration for T_3 bacteriophage at 22° C

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by charges on the phage particles, a diffusion experiment was made in the presence of 0.8 to 0.9 M NaCl. This high concentration of electrolyte did not change the diffusion rate of T4 significantly as can be seen in Fig. 5 and Table II.

TABLE I

DIFFUSION OF T3 PHAGE FOR 20 HOURS AT 4°C AT VARIOUS CONCENTRATIONS

Dilution of Stock Solution	C_o	C	C/C_o
10^5	$197 \cdot 10^3$*	$73 \cdot 10^2$	0.037
10^4	$174 \cdot 10^4$	$68 \cdot 10^2$	0.039
10^3	$195 \cdot 10^6$	$65 \cdot 10^5$	0.033
10^1	$230 \cdot 10^7$	$75 \cdot 10^6$	0.033
10^0	$223 \cdot 10^8$	$41 \cdot 10^7$	0.018
10^3	$224 \cdot 10^5$	$64 \cdot 10^4$	0.029
10^1	$282 \cdot 10^7$	$73 \cdot 10^6$	0.026
10^1	$247 \cdot 10^7$	$77 \cdot 10^6$	0.031
10^0	$218 \cdot 10^8$	$35 \cdot 10^7$	0.016

* $197 \cdot 10^3$ signifies the estimated number of phage particles per ml, and that 197 plaques were counted to make the estimate.

TABLE II

DIFFUSION OF T4 PHAGE IN 0.9 M NaCl INTO 0.8 M NaCl

t (h)	C_o	C
1:15	$50 \cdot 10^6$	$193 \cdot 10^3$
2:00	$55 \cdot 10^6$	$49 \cdot 10^4$
4:20	$52 \cdot 10^6$	$36 \cdot 10^4$
9:00	$57 \cdot 10^6$	$75 \cdot 10^4$
25:00	$55 \cdot 10^6$	$143 \cdot 10^4$
49:00	$62 \cdot 10^6$	$191 \cdot 10^4$

TABLE III

DIFFUSION OF T3 PHAGE PURIFIED BY ULTRACENTRIFUGATION. T = 4° C

t (h)	C_o	C	C/C_o	D
4:00	$40 \cdot 10^6$	$46 \cdot 10^4$	0.012	
16:00	$35 \cdot 10^6$	$108 \cdot 10^4$	0.031	
20:00	$46 \cdot 10^6$	$142 \cdot 10^4$	0.031	$3.7 \cdot 10^{-7}$ (Av.)

Diffusion of Purified Virus. It was thought that the high diffusion rate of the virus at low concentration might be altered by using ultracentrifugally purified phage. In this experiment, T3 phage in 200 ml solution, having a particle count of $4 \cdot 10^{10}$ particles/ml, was sedimented twice at 15.000 rpm in the air-driven ultracentrifuge and the final, pellet dissolved in 2 ml saline; the particle count was now $29 \cdot 10^{11}$. 0.01 ml of this solution was diluted in a 0.02 M phosphate buffer of p_H 7.4 containing 1.7% NaCl and diffused into 0.02 M phosphate buffer p_H 7.4 containing 0.85% NaCl. Both solutions were filtered

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for sterility. The particle count of the filtrate to be used for diffusion was now $64 \cdot 10^5$ particles/ml. This solution was used for diffusion at 4° C into its buffer for various times. In Fig. 4 and Table III the results are given. As can be seen, the diffusion rate is still very high and the purification process had no detectable effect on it.

Diffusion of Diffusate of T3. In this experiment the diffusate of a previous diffusion experiment was diffused in broth in the usual way to investigate possible inhomogeneity. The resulting values of this experiment are presented in Fig. 4 and Table IV.

TABLE IV

DIFFUSION OF DIFFUSATE OF T3 PHAGE. T = 4° C

t (h)	C_o	C	C/C_o	D
4:00	$27 \cdot 10^5$	$37 \cdot 10^3$	0.014	
16:00	$35 \cdot 10^5$	$98 \cdot 10^3$	0.028	
20:00	$25 \cdot 10^5$	$79 \cdot 10^3$	0.032	$3.8 \cdot 10^{-7}$ (Av.)

DISCUSSION

The results of NORTHROP[6], when expressed in the units we have used, are of interest. In that work, concentrated solutions of a staphylococcus phage which contained 10^{12} particles per ml or more gave small diffusion values (D = about $1 \cdot 10^{-8} cm^2/sec$). When the solution was diluted to contain about 10^{11} to 10^{10} particles per ml higher diffusion constants were obtained (D = about $2 \cdot 10^{-7} cm^2/sec$). The lower limit of the range studied by NORTHROP is about the same as our upper limit (10^{10} particles per ml), and the value observed by us (D = $1.0 \cdot 10^{-7} cm^2/sec$) is about half that seen by NORTHROP. At the lower limit of concentration studied by us, where the particle count was about 10^5 per ml, the diffusion constant was calculated to be $5 \cdot 10^{-7} cm^2/sec$. The diffusion experiments of NORTHROP were carried out at 10° and ours were at 4°. NORTHROP suggested that the variation in diffusion constant was evidence of dissociation of phage molecules as a result of dilution. In NORTHROP's investigation the activity method of KREUGER was used to measure phage concentrations.

Recently HERSHEY, KIMURA, AND BRONFENBRENNER[8] have suggested that the porous disc method for determining diffusion constants is unreliable because of circulation of fluid through the disc. They have reviewed their earlier work concerning heterogeneity in size of phage particles and regard the evidence for this heterogeneity in size as being insufficient.

The work here reported again shows the variation in diffusion constants with concentration. It does not appear to be evidence for heterogeneity since centrifugally purified phage and the diffusates of diffusion experiments gave the expected values for diffusion constants.

The preliminary work published by one of us, POLSON[11] was performed on phage solutions having particle counts of 10^8–10^{10} per ml. The diffusion constants were of the order of magnitude that would be expected from the sizes of bacteriophages as shown by electron micrographs. However, it can be seen from the results reported above, that the relatively low diffusion constants previously reported are explained by the dependency on concentration.

Alternatively it is suggested that these unexpectedly high diffusion constants

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would result if the phage particles possess an independent specific motility in addition to the normal diffusion. This has not definitely been proved but additional evidence from ultrafiltration experiments has been obtained to support it.

SUMMARY

The diffusion constants of T3 and T4 bacteriophages were studied by means of the multi-chamber analytical diffusion cell. The composition of the medium had no noticeable effect on the values found, but the concentration of the phage and temperature had pronounced effects on the corrected diffusion constants. At 4° C the diffusion constant of T3 varied from $5.3 \cdot 10^{-7} cm^2/sec$ to $1.0 \cdot 10^{-7} cm^2/sec$ in the concentration range 10^5 to 10^{10} particles/ml. At 22° C the diffusion constants varied from $23 \cdot 10^{-7} cm^2/sec$ to the $3 \cdot 10^{-7} cm^2/sec$ in the concentration range 10^5 to 10^9 particles/ml. These diffusion constants are much higher than would be expected from the size observed by electron microscopy.

RÉSUMÉ

Les constantes de diffusion des bactériophages T3 et T4 ont été étudiées à l'aide de la cellule analytique de diffusion à plusieurs compartiments. La composition du milieu n'a pas d'effet notable sur les valeurs trouvées, mais la concentration des phages et la température ont des effets marquants sur la valeur des constantes de diffusion corrigées. A 4° C, la constante de diffusion de T3 varie de $5.3 \cdot 10^{-7}$ cm^2/sec à $1.0 \cdot 10^{-7}$ cm^2/sec, dans des zones de concentration allant de 10^5 à 10^{10} particules/ml. A 22° C, les constantes de diffusion varient de 23.10^{-7} cm^2/sec à 3.10^{-7} cm^2/sec pour des zones de concentration allant de 10^5 à 10^9 particules/ml. Ces constantes de diffusion sont beaucoup plus élevées que celles auxquelles on pourrait s'attendre d'après la taille des particules observées au microscope électronique.

ZUSAMMENFASSUNG

Die Diffusionskonstanten von T3 und T4 Bakteriophagen wurden mit Hilfe der analytischen "Multi-chamber" Diffusionszelle untersucht. Die Zusammensetzung des Mediums hatte keinen merkbaren Effekt auf die erhaltenen Werte, aber die Phagenkonzentration und die Temperatur hatten ausgesprochene Wirkungen auf die korrigierten Diffusionskonstanten. Bei 4° C variierte die Diffusionskonstante von T3 von $5.3 \cdot 10^{-7}$ cm^2/sec bis zu $1.0 \cdot 10^{-7}$ cm^2/sec im Konzentrationsbereich 10^5 bis 10^{10} Teilchen/ml. Bei 22° variierten die Diffusionskonstanten von $23 \cdot 10^{-7}$ cm^2/sec bis zu $3 \cdot 10^{-7}$ cm^2/sec im Konzentrationsbereich 10^5 bis 10^9 Teilchen/ml. Diese Diffusionskonstanten sind viel höher als die im Elektronenmikroskop wahrgenommene Grösse erwarten liesse.

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