THE PREPARATION AND PROPERTIES OF DESOXYPENTOSENUCLEIC ACID OF BACTERIOPHAGE T2

by

ALBERT SIEGEL* AND S. J. SINGER**

*Kerckhoff Laboratories of Biology and Gates and Crellin Laboratories of Chemistry****, California Institute of Technology, Pasadena, California (U.S.A.)*

In the course of studying the high molecular weight components released by mechanical disruption from *Escherichia coli* strain B infected with the bacteriophage *T2*, it was noted that at certain stages of infection a large amount of material with the physical and chemical characteristics of desoxypentosenucleic acid (DNA) was present among the disruption products. Further investigation showed that this material was released from intact bacteriophage particles present inside the bacteria at the time of disruption. When bacteriophage particles were subjected to the same procedure used to disrupt bacteria, it was found that substantial quantities of relatively pure DNA could easily be obtained by methods likely to leave the DNA in an undegraded state. The primary purpose of this paper is to describe the preparation of this bacteriophage DNA and its behavior in preliminary physical-chemical studies.

EXPERIMENTAL RESULTS

Preparation of Phage DNA

High titer phage lysates were prepared in a glucose synthetic medium, containing 9 parts of sterile salt solution and 1 part of sterile glucose solution. The salt solution consisted of Na_2HPO_4, 7.0 g; KH_2PO_4, 3.0 g; $MgSO_4$, 0.2 g; NaCl, 0.5 g; NH_4Cl, 1.0 g; $CaCl_2$, 15 mg; H_2O, 1 liter. Approximately 10^{10} phage particles were added to 18 liters of a bacterial suspension which had attained a concentration of *ca.* $5 \cdot 10^8$ cells per ml. The culture was maintained at 35° C with vigorous aeration for 24–36 hours at which time the concentration of infective particles had reached 2 to $6 \cdot 10^{11}$ per ml. This raw lysate was passed rapidly through a refrigerated, steam-driven, Sharples super-centrifuge running at a speed of 30,000 r.p.m. with a flow rate of $^3/_4$ liter per minute, for the purpose of removing intact bacterial cells and bacterial debris. The phage particles were then sedimented by passing the clarified lysate through the Sharples super-centrifuge running at a speed of 50,000 r.p.m. with a flow rate of 3 to 4 liters per hour. After recovery from the centrifuge bowl, the mass of phage particles was suspended in *ca.* 50 ml of 0.1 ionic strength cacodylic acid buffer (buffer I), pH 6.83, containing 0.08 *M* NaCl, 0.02 *M* Na cacodylate, and 0.0023 *M* cacodylic acid. Further purification of the phage

* Present Address: Botany Division, University of California, Los Angeles, California.
** Present Address: Chemistry Department, Yale University, New Haven, Connecticut.
*** Contribution No. 1632.

References p. 319.

preparation was achieved by two cycles of differential centrifugation in a Servall angle head centrifuge, Model SS-1, as described by KOZLOFF *et al.*[1]. The final phage concentrate consisted of about 30 ml of buffer containing between $8 \cdot 10^{12}$ and $5 \cdot 10^{13}$ phage particles per ml.

The phage suspension was next submitted for 30 minutes to the action of an Eppenbach colloid mill, Model QV-6, with the clearance between the rotor and stator set at 25–50 microns. The bypass and outlet assembly of the mill was replaced by a length of $\frac{1}{4}'' \times \frac{3}{16}''$ rubber tubing which served to decrease the minimal circulation capacity of the mill from 125 ml to 25 ml. The mill was cooled with circulating ice-water, and the temperature of the circulating phage suspension did not rise above 15° C during the course of the colloid mill action. In various runs from $\frac{1}{5}$ to $\frac{2}{3}$ of the phage particles were inactivated by the colloid mill treatment. The material which was recovered from the colloid mill is termed the phage grindate.

The sedimentation characteristics of purified phage preparations and phage grindates were examined in an analytical ultracentrifuge. Sedimentation patterns of a typical purified phage preparation are illustrated in Fig. 1a. The diagram on the right

Fig. 1. Ultracentrifuge diagrams of T2 bacteriophage preparations. 1a. Untreated phage. 1b. Phage after colloid mill treatment. Sedimentation proceeds to the left. The right-hand diagram in each experiment was taken at a rotor speed of 9,000 r.p.m.; the left at 47,520 r.p.m. The time in seconds after the rotor had attained the appropriate speed is given under each diagram. The experiments were performed in buffer I.

in Fig. 1a is taken at a rotor speed of 9,000 r.p.m., while that on the left is taken at about 50,000 r.p.m. At a rotor speed of 9,000 r.p.m., a peak due to intact phage particles sediments from the purified phage preparation, leaving behind a clear solution from which no other material sediments until the rotor speed is raised to about 50,000 r.p.m. At this speed a small sharp peak appears which in accord with other observations[1] can be attributed to a small amount of free DNA in the solution (see below). A phage grindate, when examined under the same ultracentrifugal conditions, shows a striking difference in composition. As illustrated by Fig. 1b, there is a marked diminution in the amount of material with the sedimentation velocity of intact phage particles. Furthermore, there also appear at the rotor speed of 9,000 r.p.m. new peaks sedimenting less rapidly than intact phage particles, presumably due to phage disruption products. No other peaks are observed to sediment until a rotor speed of 50,000 r.p.m. is attained. At this speed a very sharp peak is observed which is present to a much greater extent than the corresponding peak in the intact bacteriophage preparation. As will be shown below, the material represented by this peak has been isolated and identified as almost pure DNA that has been released from the phage particles as a result of colloid mill treatment.

The fact that no components were observed in the sedimentation patterns of the

References p. 319.

phage grindate at rotor speeds between 9,000 r.p.m. and 50,000 r.p.m. under the conditions of the experiment suggested that the DNA present in the phage grindate could be readily freed of other high molecular weight components by high-speed centrifugation. Accordingly, phage grindates were centrifuged in the size 40 head of a Spinco Model L preparative centrifuge at a speed of 40,000 r.p.m. for 1 hour. A pellet was deposited leaving a clear supernatant solution which upon analysis in the ultracentrifuge was found to have only the sharp peak representing the DNA component, as illustrated in Fig. 2.

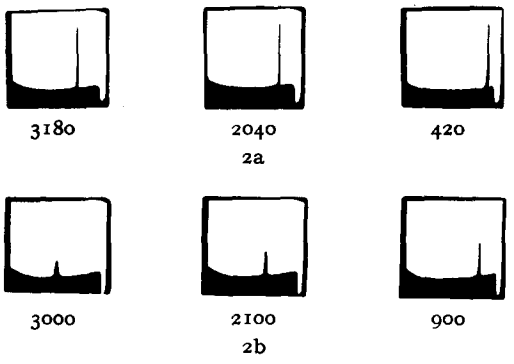

Fig. 2. Ultracentrifuge diagrams of phage DNA at different concentrations. 2a. 3.6 mg DNA/ml; 2b. 0.4 mg DNA/ml. The time in seconds after the rotor had attained the speed of 50,220 r.p.m. is given under each diagram. Both experiments were performed in buffer I.

Chemical characterization of phage DNA preparations

Bacteriophage T2 contains approximately 40% DNA[2]. Although our yield data are not quantitative, it appears that from 10 to 25% of the DNA of the phage subjected to colloid-milling was recovered at the end of the experiment. In view of the incomplete disruption of the phage particles, and of the losses in material incurred during colloid-milling, these data suggest that a considerable proportion of the phage DNA is released by this method of preparation.

One batch of phage grindate No. 61 after high speed centrifuging produced 25 ml of a clear supernatant solution containing 3.6 mg DNA/ml, as estimated by the Dische cysteine hydrochloride test[3]. A sample of purified squid testis DNA, kindly supplied by A. RICH of this Institute, was used as a standard in the colorimetric determinations. The dry weight of the solution, corrected for the presence of buffer salts, proved also to be 3.6 mg/ml. The material after prolonged dialysis against buffer I contained 13.9% nitrogen and 7.2% phosphorus, leading to an N/P ratio of 1.9. Nitrogen was estimated by the Nessler method, phosphorus by the Allen method[4]. The ultraviolet absorption spectrum was closely similar to that of thymus DNA[5]. It exhibited a maximum optical density at 260 mμ and a minimum at 232 mμ. The ratio of the extinction coefficients at these two wave lengths was 2.3. Another preparation of phage DNA (# 67) was subjected to the Folin-Ciocalteu phenol test. The solution, at a concentration of 3.6 mg DNA/ml, contained $2.2 \cdot 10^{-2}$ mg tyrosine/ml, if all the color developed in the test is attributed to tyrosine. Assuming that the tyrosine was a constituent of protein, and that the protein contains 4.6% tyrosine[2], this preparation of phage DNA contained about 13% protein.

References p. 319.

An attempt was made to reduce the small amount of protein contaminant by further high speed centrifugation at 40,000 n.p.m. for 2 hours. The top one-half of the supernatant solution was carefully removed and analysis revealed the presence of 2.3 mg DNA/ml and $1.2 \cdot 10^{-2}$ mg tyrosine/ml, or about 11% protein. Since the DNA was partially sedimented in this centrifugation without appreciably reducing the per cent. tyrosine content of the supernatant solution, it appears that the protein sedimented at a rate close to that of DNA, and may be bound to the DNA. The amount of protein, however, was small, and further purification of the DNA was not attempted.

Sedimentation behavior of phage DNA preparations

Phage DNA of 87% purity as judged from chemical analysis was examined in the ultracentrifuge in buffer I at a number of different concentrations. Diagrams of two representative experiments are illustrated in Fig. 2 where it is evident that the velocity of sedimentation is strongly dependent on concentration. Calculated sedimentation constants at different concentrations are listed in Table I. When the reciprocal of the sedimentation constant is plotted against concentration as in Fig. 3, a straight line may be drawn through the points at lower concentrations although there is a definite indication of downward curvature at higher concentration. This method of extrapolating high-concentration-dependent sedimentation constants to infinite dilution[6] results in an extrapolated sedimentation constant of $S^{w}_{20} = 13.1\,S$, and the result is in accord with previous investigations of thymus DNA[7,8,9].

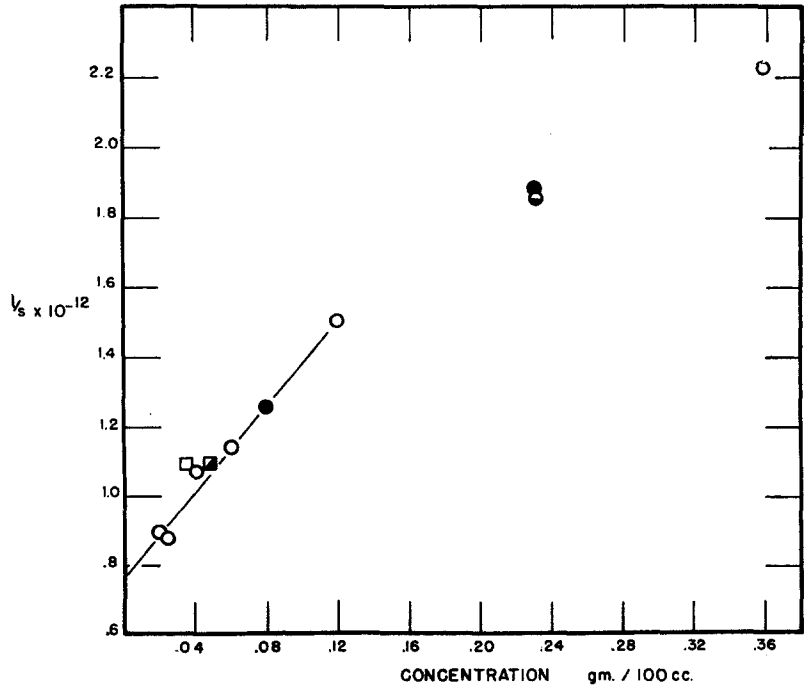

Fig. 3. $1/s$ as a function of concentration for various DNA samples: ○ phage DNA #61; ◒ phage DNA #67; □ bacterial DNA; ◪ bacterial DNA + phage DNA; ● phage DNA + squid testis DNA.

References p. 319.

TABLE I

DNA SEDIMENTATION DATA

Type of DNA	*DNA Concentration mg/ml*	$s^{w}_{20} \times 10^{13}$
A. Bacteriophage T2, #61	3.58	4.5
	1.19	6.6
	0.60	8.7
	0.40	9.3
	0.24	11.3
	0.20	11.1
	0.00**	13.1**
B. Bacteriophage T2, #67	2.31*	5.4
C. Bacterial DNA	0.57	9.1
D. ½ A plus ½ C	0.59	9.1
E. ½ A plus ½ Squid Testis DNA	2.29	5.3
	0.76	7.9

* Performed in buffer II. All other experiments performed in buffer I.
** Extrapolated.

Electrophoretic behavior of phage DNA preparations

Phage DNA, prepared by the method described above, was examined in a Perkin-Elmer, Model 38, electrophoresis apparatus. The results are shown in Fig. 4 where it is evident that only one peak appears in the scanning pattern. The experiment was performed in 0.2 ionic strength cacodylic acid buffer (buffer II).

The mobility of the peak in the descending limb is $-17.5 \cdot 10^5$ cm²/sec/volt.

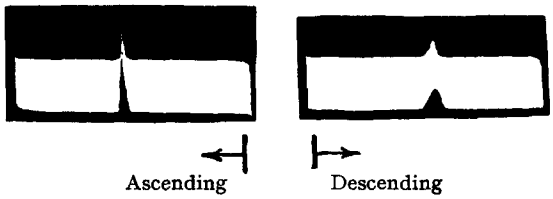

Fig. 4. Electrophoresis diagrams of purified bacteriophage DNA. The starting boundaries are indicated by arrows. The migration time was 4020 seconds at a potential gradient of 4.1 volts/cm. The concentration was 0.72 mg DNA/ml in buffer II.

A comparison of bacterial and phage DNA

During the course of an investigation of the effect of bacteriophage infection upon the soluble high molecular weight components of *E. coli*, which will be the subject of a forthcoming communication, a bacterial DNA component was revealed by ultracentrifugal and electrophoretic analysis. The question arose as to whether there was a difference in the physical chemical characteristics of the bacterial and phage DNA's. SMITH AND WYATT[10] have recently reported differences in the nitrogenous base composition of DNA preparations from *E. coli* and T2 bacteriophage particles. DNA was extracted from cells of *E. coli* by the following method. Eighteen liters of nutrient broth, containing bacteria in the logarithmic phase of growth, at a concentration of $2 \cdot 10^8$ cells per ml, were passed through the Sharples super-centrifuge running at a speed of 30,000 r.p.m. with a flow rate of $^3/_4$ of a liter per minute. The bacterial mass of 10–12 g

References p. 319.

wet weight, scraped from the bowl of the centrifuge, was washed twice in buffer I and after resuspension in a small volume of buffer the bacteria were submitted to colloid mill action under the same conditions as described for phage disruption. The resultant bacterial grindate was centrifuged at 40,000 r.p.m. for 5 hours leaving a supernatant containing free DNA as well as some of the other bacterial high molecular weight components. The DNA was further purified by the method of SEVAG, LACKMAN AND SMOLLENS[11]. The solution, partially purified by centrifugation, was shaken vigorously with an equal part of a solution consisting of 35 parts of chloroform to 10 parts of amyl alcohol. This process was repeated 16 times until only a trace of chloroform-protein gel complex was still being formed. The aqueous layer was subjected to negative pressure to remove the organic solvents and then dialyzed against buffer II overnight. The resultant purified solution contained 1.7 mg DNA/ml.

The behavior of bacterial DNA during ultracentrifugation and electrophoresis

When the bacterial DNA preparation was examined in the electrophoresis apparatus, two major components were observed as illustrated in Fig. 5b. In the scanning pattern of the descending limb 60% of the total area is present under a peak with mobility $-17.5 \cdot 10^{-5}$ cm²/sec/volt and 40% under a peak with mobility $-13.7 \cdot 10^{-5}$ cm²/sec/volt. The ratio of the area under the faster moving peak to the DNA concentration of the solution is the same as that found for phage DNA preparations. This observation coupled with the fact that the mobility of the faster moving peak is the same as that of phage DNA leads to the conclusion that the faster moving peak represents bacterial DNA.

Ultracentrifugal analysis of the bacterial DNA preparation gave results, Fig. 5a, in agreement with those obtained by electrophoresis. The typical spike-like DNA peak is seen prominently in the sedimentation diagram. Another component is also present which has a smaller sedimentation constant and is apparently heterogenous.

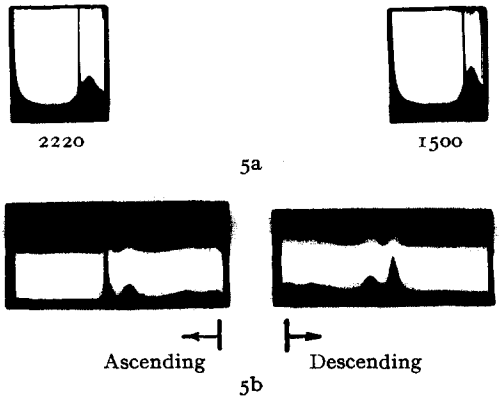

Fig. 5. Ultracentrifuge and electrophoresis diagrams of a bacterial DNA preparation. 5a. Ultracentrifuge diagrams. The time in seconds after the rotor had attained the speed of 50,220 r.p.m. is given under each diagram. 5b. Electrophoresis scanning patterns. The migration time was 4020 seconds at a potential gradient of 4.1 volts/cm.

Mixture experiments with bacterial DNA and phage DNA

Since the DNA's extracted from the virus and from the uninfected bacterial host

References p. 319.

seemed to behave very similarly by our methods of analysis, a study was initiated to test whether the two DNA's could be separated from a mixture by electrophoresis and analytical ultracentrifugation. Sedimentation experiments were performed with mixtures of equal parts of bacterial and phage DNA's at two different total DNA concentrations, 1.8 mg/ml and 0.6 mg/ml. Ultracentrifugal resolution of the two DNA's did not occur, as is evident in Fig. 6a, and the sedimentation constant of the mixed DNA peak corresponded to that of bacteriophage DNA alone at the same total DNA concentration. Electrophoresis experiments with mixtures of equal parts of the two DNA preparations were performed at pH 6.73, and pH 4.20. No resolution of the DNA peak was observed (Fig. 6c and 6d). In acetate buffer at pH 4.20 and ionic strength 0.2, containing 0.1 *M* NaCl (buffer III), the descending mobility of the DNA peak is $-17.2 \cdot 10^{-5}$ cm^2/sec/volt.

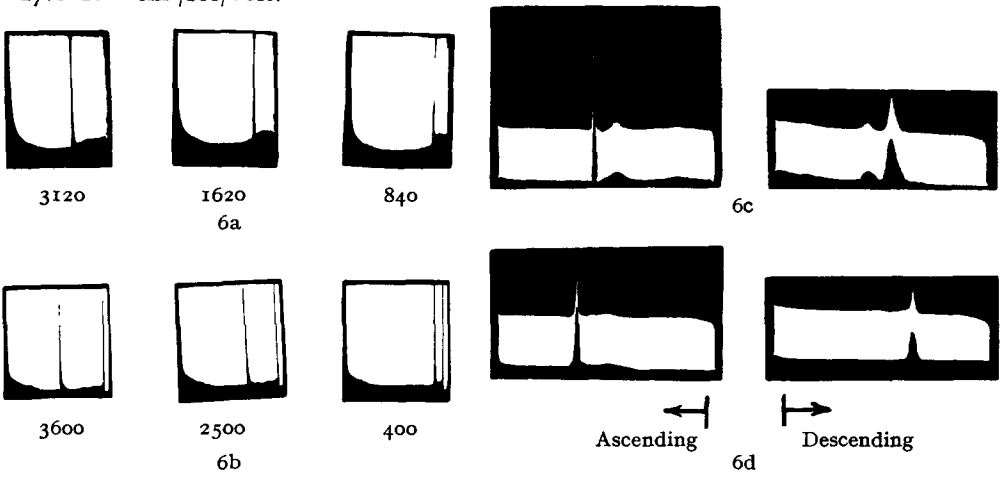

Fig. 6. The sedimentation and electrophoretic behavior of mixtures of several DNA preparations. 6a. Ultracentrifuge diagrams of phage DNA and bacterial DNA. 6b. Ultracentrifuge diagrams of phage DNA and squid testis DNA. The time in seconds after the rotor had attained the speed of 50,220 r.p.m. is given under each ultracentrifuge diagram. 6c. Electrophoresis pattern of phage DNA and bacterial DNA at pH 6.7 in buffer II. The experiment ran for 4020 seconds at a potential gradient of 4.1 volts/cm. 6d. Electrophoresis pattern of phage DNA and bacterial DNA at pH 4.2 in buffer III. The experiment ran for 4620 seconds at a potential gradient of 4.4 volts/cm.

Comparison of phage DNA and squid testis DNA.

In view of the inability to distinguish between bacterial DNA and phage DNA by physical chemical means, it was decided to explore the significance of these observations by studying mixtures of phage DNA with a DNA from a totally unrelated source. Accordingly, mixtures of about equal parts of phage DNA and purified squid testis DNA, kindly supplied by Dr A. RICH of this Institute, were examined in the ultracentrifuge at two total DNA concentrations, 2.3 mg/ml and 0.8 mg/ml, in buffer I. Again, no resolution of the two DNA's was achieved (Fig. 6b) and the sedimentation constants of the mixed DNA peak had values, given in Table I, which corresponded to values predicted from the plot presented in Fig. 3.

DISCUSSION

The method for preparing T2 bacteriophage DNA, described in this paper, is

References p. 319.

characterized by the ability to prepare considerable quantities of DNA in a relatively pure state without removing it from an aqueous medium and without the use of any chemical reagent other than a neutral buffer. Such a procedure has the advantage that there is less probability of the DNA molecules being altered chemically or structurally in some unknown manner during the course of purification. Phage disruption products other than DNA have not as yet been characterized. The ability to recover appreciable quantities of these products, however, by our methods, opens the door to a more revealing study of the composition of virus than has hitherto been possible by a study of preparations of intact bacteriophage particles.

ANDERSON[12] and ANDERSON *et al.*[13] have demonstrated that when even-numbered T bacteriophage strains are rapidly diluted from a concentrated salt solution or subjected to intense sonic vibrations, they are inactivated. The treated suspensions, when viewed in the electron microscope, reveal the presence of phage "ghosts". These ghosts consist of a thin membrane with the tail still attached, but the usual internal morphology associated with the head is absent. The chemical constitution of the disruption products, however, was not investigated. Whether the methods used by ANDERSON disrupt the bacteriophage organization in the same manner as the method described in this paper is still to be investigated. DULBECCO[14] has recently observed that nucleic acid is released into the medium when phage particles are inactivated by high doses of ultraviolet light.

Most previous physical chemical studies of DNA have been performed with material isolated from thymus. These investigations have indicated that the sedimentation constant of DNA is strongly dependent on concentration, resulting in very sharp peaks in the ultracentrifuge. Three recent investigations of thymus DNA prepared under similar conditions have yielded similar values for the extrapolated value of S^{w}_{20}: 12.5 *S*[7], 12.6 *S*[8], and 13.2 *S*[9]. The value obtained for bacteriophage DNA in this study, 13.1 *S*, is closely similar to those reported for thymus DNA. Furthermore, mixture experiments suggest that bacterial DNA and squid testis DNA are characterized by the same extrapolated sedimentation constant as the bacteriophage DNA. While it must be recognized that the sedimentation constant of long thread-like molecules, such as DNA, does not vary rapidly with molecular weight, and furthermore that the strong concentration dependence of the sedimentation constant may prevent the resolution of species of DNA of slightly different sedimentation constants, nevertheless the results which we have obtained suggest that the molecular weights of DNA's obtained from widely different sources may be remarkably similar.

Electrophoretic mobility under a given set of conditions is a function of the charged chemical groups of a substance, and it is therefore not surprising that the chemically similar DNA's have similar mobilities. It may be, however, that small differences in mobility do exist between different DNA's. Thymus DNA has a mobility of about $-13 \cdot 10^{-5}$ cm^2/sec/volt in acetate buffer at pH 4.2, ionic strength 0.1, definitely smaller than the mobility at pH 7.0[15]. On the other hand, our results indicate that phage DNA and bacterial DNA have the same mobilities at these two pH values in buffers of ionic strength 0.2. It would be of interest to investigate more fully whether the electrophoretic mobilities of DNA's from different sources can be distinguished. Methods have been developed for determining the nitrogenous base composition of DNA[16], and DNA's from a variety of sources have been examined[16,10]. It is possible, therefore, to study how the chemical composition of DNA preparations, whose degree of purity is precisely determinable, affects their electrophoretic mobility.

References p. 319.

SUMMARY

1. A simple method is presented for the isolation of DNA of about 90% purity from bacteriophage T2 without the use of chemical reagents other than a neutral buffer, which results in a product which is homogeneous electrophoretically and ultracentrifugally.

2. In a cacodylate buffer of 0.1 ionic strength and pH 6.83, phage DNA has an extrapolated sedimentation constant of $S^{w}_{22} = 13.1$ S.

3. The DNA of *E. coli* has been partially purified and its physical chemical properties compared with those of phage DNA. The two DNA's cannot be resolved in a mixture by ultracentrifugation or electrophoresis under the conditions investigated.

4. The sedimentation properties of phage DNA, bacterial DNA, and squid testis DNA are apparently indistinguishable, and very similar to those reported for thymus DNA. It is suggested therefore that the molecular weights of these DNA's from widely different sources are similar.

RÉSUMÉ

1. Nous décrivons une méthode simple pour isoler du ADN d'une pureté d'environ 90% à partir de bactériophage T2 sans emploi d'agents chimiques autre qu'un tampon neutre. Par cette méthode l'on obtient un produit qui se montre homogène à l'étude électrophorétique et dans l'ultracentrifuge.

2. Dans un tampon cacodylate de force ionique 0.1 et de pH 6.83, la constante de sédimentation extrapolée de ADN de phage est $S^{w}_{22} = 13.1$ S.

3. Nous avons partiellement purifié du ADN de *E. coli* et nous en avons comparé les propriétés physico-chimiques à celles du ADN de phage. Dans les conditions étudiées nous n'avons réussi, ni par ultracentrifugation ni par électrophorèse, à séparer les deux ADN à partir de leur mélange.

4. Les propriétés de sédimentation d'ADN de phage, de bactérie et de testicule de seiche ne peuvent apparemment être distinguées entre elles et ressemblent beaucoup à celles décrites pour l'ADN de thymus. C'est pourquoi nous supposons que les poids moléculaires de ces ADN de sources très différentes sont semblables.

ZUSAMMENFASSUNG

1. Es wird eine einfache Methode zur Isolierung von DNS in einer Reinheit von ungefähr 90% aus Bakteriophage T2 ohne den Gebrauch anderer chemischer Reagenzien als den eines neutralen Puffer vorgeschlagen, die ein Produkt ergibt, das sich bei elektrophoretischen Untersuchungen und in der Ultrazentrifuge als homogen erweist.

2. In einem Kakodylatpuffer mit Ionenstärke 0.1 und pH 6.83 hat die Phagen-DNS eine extrapolierte Sedimentationskonstante von $S^{w}_{22} = 13.1$ S.

3. Die DNS von *E. coli* wurde partiell gereinigt und ihre physikalisch-chemischen Eigenschaften mit denjenigen von Phagen-DNS verglichen. Die zwei DNS's können nicht aus ihrer Mischung mit der Ultrazentrifuge oder durch Elektrophorese unter den untersuchten Bedingungen getrennt werden.

4. Die Sedimentationseigenschaften der Phagen-DNS, der Bakterien-DNS und der DNS aus Tintenfischtestikeln sind offensichtlich nicht unterscheidbar und sehr ähnlich denen, die für Thymus-DNS berichtet wurden. Es wird daher vermutet, dass die Molekulargewichte dieser DNS's aus sehr verschiedenen Quellen ähnlich sind.

REFERENCES

1 L. M. KOZLOFF AND F. W. PUTNAM, *J. Biol. Chem.*, 181 (1949) 207.
2 S. S. COHEN, *J. Exptl. Med.*, 84 (1946) 521.
3 Z. DISCHE, *Proc. Soc. Exptl. Biol. Med.*, 55 (1944) 217.
4 R. J. L. ALLEN, *Biochem. J.*, 34 (1940) 858.
5 A. MIRSKY AND A. W. POLLISTER, *J. Gen. Physiol.*, 30 (1946) 117.
6 N. GRALEN, Dissertation, Uppsala, 1944.
7 H. KAHLER, *J. Phys. Coll. Chem.*, 52 (1948) 676.
8 R. CECIL AND A. G. OGSTON, *J. Chem. Soc.*, 150 (1948) 1382.
9 A. G. OGSTON, in E. R. BLOUT AND P. M. DOTY, *Science*, 112 (1950) 639.
10 J. D. SMITH AND G. R. WYATT, *Biochem. J.*, 49 (1951) 144.
11 M. G. SEVAG, D. B. LACKMAN AND J. SMOLENS, *J. Biol. Chem.*, 124 (1938) 425.
12 T. F. ANDERSON, *Bot. Rev.*, 15 (1949) 464.
13 T. F. ANDERSON, S. BOGGS AND B. C. WINTERS, *Science*, 108 (1948) 18.
14 R. DULBECCO, Personal communication.
15 E. STENHAGEN AND T. THEORELL, *Trans. Faraday Soc.*, 35 (1939) 743.
16 E. CHARGAFF, S. ZAMENHOF AND C. GREEN, *Nature*, 165 (1950) 756.

Received August 5th, 1952