*A DYE-BUOYANT-DENSITY METHOD FOR THE DETECTION AND ISOLATION OF CLOSED CIRCULAR DUPLEX DNA: THE CLOSED CIRCULAR DNA IN HELA CELLS**

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Covalently closed circular duplex DNA's are now known to be widespread among living organisms. This DNA structure, originally identified in polyoma viral DNA,[1, 2] has been assigned to the mitochondrial DNA's in ox[3] and sheep heart,[4] in mouse and chicken liver,[3] and in unfertilized sea urchin egg.[5] The animal viral DNA's—polyoma, SV40,[6] rabbit[7] and human[8] papilloma—the intracellular forms of the bacterial viral DNA's—ϕX174,[9, 10] lambda,[11, 12] M13,[13] and P22[14]—and a bacterial plasmid DNA, the colicinogenic factor E_2,[15] have all been shown to exist as closed circular duplexes. Other mitochondrial DNA's[16, 17] and a portion of the DNA from boar sperm[18] have been reported to be circular, but as yet have not been shown to be covalently closed.

The physicochemical properties of closed circular DNA differ in several respects from those of linear DNA or of circular DNA containing one or more single-strand scissions.[19] The resistance to denaturation,[2, 20] the sedimentation velocity in neutral and alkaline solution, and the buoyant density in alkaline solution are all enhanced in the closed circular molecules. These three effects are a direct consequence of the topological requirement that the number of interstrand cross-overs must remain constant in the closed molecule.

The principal methods currently used for the detection and the isolation of closed circular DNA are based on the first two general properties. In this communication we describe a method based on the buoyant behavior of closed circular DNA in the presence of intercalating dyes.

The binding of intercalative dyes has recently been shown to cause a partial unwinding of the duplex structure in closed circular DNA.[22–24] In such molecules any unwinding of the duplex causes a change in the number of superhelical turns, so that the total number of turns in the molecule remains constant. A small and critical amount of dye-binding reduces the number of superhelical turns to zero. Further dye-binding results in the formation of superhelices of the opposite sign or handedness. The creation of these new superhelices introduces mechanical stresses into the duplex and a more ordered conformation into the molecule. These effects increase the free energy of formation of the DNA-dye complex. The maximum amount of dye that can be bound by the closed molecule is therefore *smaller* than by the linear or nicked circular molecule. Correspondingly, since the buoyant density of the DNA-dye complex[23, 25] is inversely related to the amount of dye bound, the buoyant density of the closed circular DNA-dye complex at saturation is *greater* than that of the linear or nicked circular DNA-dye complex.[23] Bauer and Vinograd have shown that the above effect results in a buoyant density difference of approximately 0.04 gm/ml in CsCl containing saturating amounts of ethidium bromide, an intercalating dye extensively studied by Waring[26] and Le Pecq.[27]

The method has been tested with known mixtures of nicked and closed circular viral DNA's, and has been used to isolate closed circular DNA from the mitochondrial fraction of HeLa cells and from extracts of whole HeLa cells.

Materials and Methods.—Preparation of HeLa cell extracts: HeLa S3 cells were grown on Petri dishes in Eagle's medium containing 10% calf serum. H^3-thymidine, 18 c/mmole, was obtained from the New England Nuclear Corporation. Ten μc per ml of medium were added to each plate 20 hr before the cells were harvested. After washing with isotonic buffer and decanting, the cells were treated by the method described by Hirt[28] for the separation of polyoma DNA from nuclear DNA. Approximately 2 ml of 0.6% sodium dodecylsulphate (SDS), in 0.01 *M* ethylenediaminetetraacetate (EDTA), 0.01 *M* tris, pH 8, were added to the plates. After 30 min at room temperature, the viscous extracts were gently scraped from the plates with a rubber policeman and transferred with a widemouth pipet to a centrifuge tube. Either 5 *M* NaCl or 7 *M* CsCl was then added with gentle mixing to a final salt concentration of 1 *M*. The resulting solution was cooled to 4° and centrifuged for 15 min at 17,000 × *g* in a Servall preparative ultracentrifuge. The supernatant solution was dialyzed at 4° overnight against two changes of 0.01 *M* EDTA, 0.01 *M* tris, pH 8 buffer in order to remove H^3-thymidine. The mitochondria from HeLa cells were isolated by differential centrifugation of an homogenate followed by banding in a sucrose gradient.

Preparation of viral DNA: Polyoma viral DNA was prepared as described previously.[19] The intracellular lambda DNA was kindly supplied by John Kiger and E. T. Young, II, of the Biology Division.

Chemicals: The ethidium bromide was obtained as a gift from Boots Pure Drug Co., Ltd., Nottingham, England. Harshaw optical grade cesium was used. The SDS was obtained from Matheson Company. All other chemicals were of reagent grade.

Preparative ultracentrifugation: The experiments were performed in SW50 rotors in a Beckman Spinco model L preparative ultracentrifuge at 20°C. The CsCl solutions, in either cellulose nitrate or polyallomer tubes, were overlaid with light mineral oil. After centrifugation, the tubes were fractionated with a drop-collection unit obtained from Buchler Instruments. The drops were collected in small vials or on Whatman glass-fiber GF/A filters. The dried filters were immersed in 10 ml of toluene-PPO-POPOP, and the samples were counted in a Packard Tri-Carb scintillation counter.

Electron microscopy: Specimens for electron microscopy were prepared by the method of Kleinschmidt and Zahn[29] and were examined in a Phillips EM200 electron microscope. All electron micrographs were made at a magnification of ×5054. The magnification factor was checked with a grating replica. Cytochrome *c* was obtained from the California Biochemical Corporation.

Fluorescence: Prior to drop collection, the centrifuge tubes were examined in a darkened room with 365 mμ light from a Mineral Light Lamp or preferably from a "Transilluminator" supplied by Ultraviolet Products, Inc., San Gabriel, California. The tubes were photographed on Polaroid type-146L film through a "contrast filter" from Ultraviolet Products, Inc. The fluorescence measurements were performed with a double-monochromator apparatus constructed in this laboratory by W. Galley and N. Davidson. The instrument was calibrated with solutions of ethidium bromide (5×10^{-3} to 1.0 μg/ml) in calf thymus DNA, 20 μg/ml, 1 *M* CsCl. The intensity of fluorescence was measured at 590 mμ with an exciting wavelength of 548 mμ.[30]

Results.—Selection of initial dye concentration, cesium chloride concentration, and centrifugation variables: The conditions for the experiments in the swinging-bucket rotor in the model L ultracentrifuge were selected with the object of obtaining separations similar to those obtained at equilibrium within a reasonable period of time. At equilibrium, the separation between components is approximately constant at initial dye concentrations between 50 and 100 μg/ml.[23] At these dye levels the buoyant densities are between 1.57 and 1.62 gm/ml. Figure 1 presents the dye and density distributions in CsCl solutions centrifuged for 24 and 48 hours. The initial dye concentration in both cases was found at a distance from the meniscus corresponding to four-tenths the length of the liquid column. In

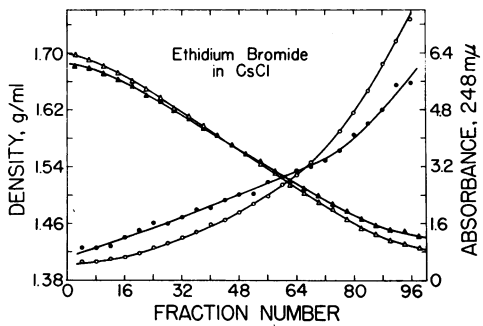


FIG. 1.—Density and dye distributions in CsCl density gradient columns, 3.00 ml 1.550 gm/ml CsCl, 100 μg/ml ethidium bromide, 24 (●, ▲) and 48 (○, △) hr at 43 krpm in a SW50 rotor at 20°.

this region of the cell, the time dependence of the dye concentration is minimal, and the constant density gradient is equal to approximately 0.10 gm/cm⁴.

Results with purified mixtures of nicked and closed circular DNA: Figure 2 presents the results obtained with 1.5 μg of tritiated polyoma DNA. Substantially complete resolution was obtained with this DNA which has a molecular weight of three million daltons. Since the fractional amount of closed circular material corresponded to the fraction obtained in analytical sedimentation velocity analyses,[21] it is concluded that single-strand scissions did not occur during the course of the experiments. An experiment in which the DNA was contained in a thin lamella at the top of the liquid column was performed to test the effect of the initial DNA distribution. The results were substantially the same as those obtained when the DNA was uniformly distributed in the CsCl solution.

The results obtained with a mixture of approximately 60 μg of covalently closed and nicked circular lambda DNA, mol wt = 3×10^7, are presented in Figure 3.

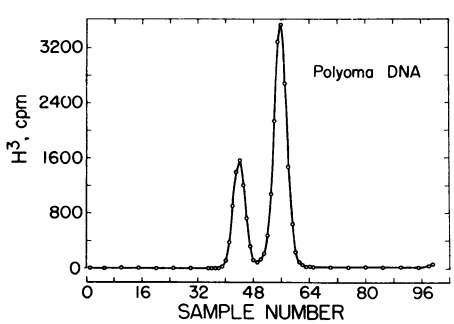


FIG. 2.—A mixture of purified polyoma DNA I and II 1.5 μg in buoyant CsCl, 3.00 ml 1.566 gm/ml CsCl, 100 μg/ml ethidium bromide, 24 hr at 43 krpm, 20°. The band maxima were separated by 12 fractions (four 7.5-μl drops per fraction). The buoyant densities of I and II are 1.588 and 1.553 gm/ml, respectively. The sample contains 30% I as indicated above compared with 32% by analytical band centrifugation.[21]

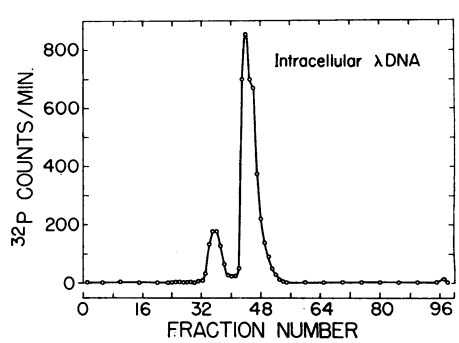


FIG. 3.—A mixture of purified intracellular lambda DNA I and II, 3.00 ml 1.55 gm/ml CsCl, 100 μg/ml ethidium bromide, 24 hr at 43 krpm, 20°. The centroids are separated by 9.5 fractions and 0.31 ml. Component I accounts for 16% of the total counts.

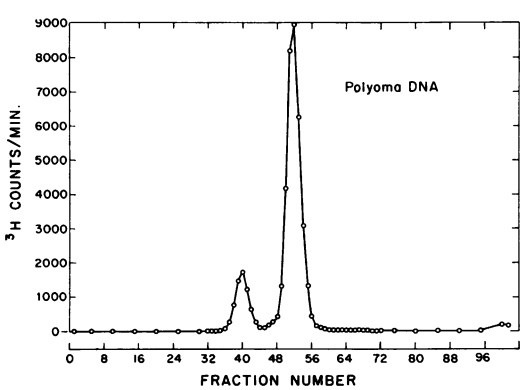
In this experiment, the nicked circular DNA formed a band of relatively high viscosity that may have distorted and broadened the light band during drop collection. An experiment to determine the effect of ethidium bromide on the protoplast assay for lambda DNA[32] was performed in collaboration with J. Kiger and E. T. Young, II. It was found that linear lambda had a normal titer when diluted

by a factor of 1000 from a solution containing ethidium bromide and CsCl at the concentrations which occur at band center.

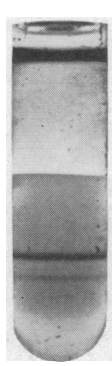
Detection of DNA by absorbance, fluorescence, and scintillation counting: The data in Figures 2 and 3 were obtained by scintillation counting of dried filter papers containing dye, cesium chloride, and labeled DNA. To examine the effect of the presence of the dye upon counting efficiency, a series of sixteen 50-μl samples of H^3-thymidine in a 1.55 gm/ml CsCl solution containing ethidium bromide in concentrations varying from 0 to 286 μg/ml were counted. The relative counting efficiency decreased linearly with a least-squares slope of 8.6×10^{-4} ml/μg. The dye gradient thus caused a difference of 1 per cent in relative counting efficiency between the two bands in Figure 2, while the dye depressed the relative counting efficiency by 8 per cent.

Red bands containing about 5 μg of DNA can be observed visually in the centrifuge tube. If an adequate amount of DNA is present, the fractionated gradient may be assayed spectrophotometrically at 260 mμ. At saturation, the increase in absorbance caused by dye-binding is about 40 per cent with linear DNA and about 20 per cent with closed circular DNA.

The photograph in Figure 4 shows the fluorescent emission from two DNA bands. The fluorescence from DNA in amounts as low as 0.5 μg per band are detectable visually. Since the separation between the two bands corresponds reliably to 0.30–0.36 ml, it was possible to use the fluorescent, less dense band as a marker to locate closed circular DNA present in amounts below the limit of detectability by spectroscopic or radioactive procedures. The method is thus especially suitable for examining nonradioactive preparations from tissues of higher animals.



(a)



(b)

FIG. 4.—A mixture of purified polyoma DNA I and II, 3.00 ml, 1.558 gm/ml CsCl, 100 μg/ml ethidium bromide, 48 hr at 43 krpm, 20°. (*a*) The band maxima are separated by 12 fractions and 0.36 ml. The calculated buoyant densities are 1.592 and 1.556 gm/ml. (*b*) A photograph of the centrifuge tube prior to drop collection. The tube contains a total of 4 μg of DNA, and 0.64 μg of component I.

Removal of ethidium bromide from DNA solutions: It is often desirable to remove the dye quantitatively from a DNA sample. This was accomplished in a single passage of 1.0 ml of polyoma I DNA (40 μg/ml DNA, 100 μg/ml dye, 1 *M* CsCl) through a 0.8×4.5-cm column of analytical grade Dowex-50 resin. The fractions containing DNA were consolidated and were found to contain less than 1×10^{-2} μg dye in a fluorometric analysis.

Isolation of closed circular DNA from H^3-thymidine-labeled HeLa cells: Hela cell monolayers in Petri dishes containing 10^7 cells were treated with SDS by the pro-

cedure described by Hirt.[28] The results obtained when the extract was purposely sheared and when the shear was minimized (Figs. 5*a* and *b*) show that the relative amount of material in the light band was decreased when shear was minimized. The dense band in Figure 5*a* is clearly contaminated by material from the light band and reprocessing the dense band in a second CsCl-dye gradient would be necessary to obtain resolved bands.

Electron-microscope examination of the circular DNA in the dense band of HeLa cell extracts: Examination of electron micrographs of specimens prepared from fractions 33–36 in the dense band of HeLa cell extracts (Fig. 5*c*) showed less than 0.1

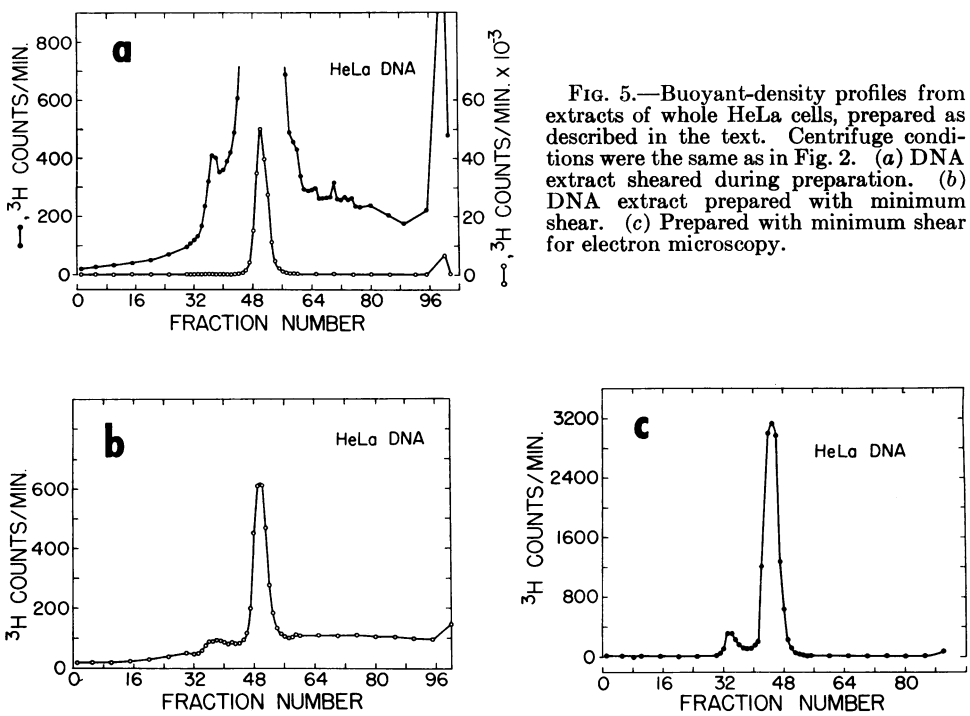


FIG. 5.—Buoyant-density profiles from extracts of whole HeLa cells, prepared as described in the text. Centrifuge conditions were the same as in Fig. 2. (*a*) DNA extract sheared during preparation. (*b*) DNA extract prepared with minimum shear. (*c*) Prepared with minimum shear for electron microscopy.

per cent of distinguishably linear DNA. The light band contained long linear DNA. The dense band contained an array of circles in three size groups: a homogeneous group of molecules with a mean length of 4.81 ± SE 0.24 microns; a heterogeneous population with lengths from 0.2 to 3.5 microns; and a paucidisperse set 2–4 times the length of the DNA in the homogeneous group. Figure 6 presents micrographs selected to illustrate the three size classes, and Figure 7 shows the frequency distribution among the first two groups. A survey of several hundred molecules on sparsely populated specimen grids revealed that the frequencies of the small and the large size classes were each about one-tenth the frequency of the homogeneous size class. In the large size class, the dimers were observed more frequently than the trimers or tetramers. The DNA from mitochondria isolated from HeLa cells consisted principally of molecules in the homogeneous size group. We conclude, therefore, that the homogeneous size class in Figure 7 is of mito-

chondrial origin. Small circular DNA's were not observed in significant amounts in the preparation of DNA from the isolated mitochondria.

Discussion.—The method described in this paper has proved to be a simple and direct procedure for obtaining closed circular DNA from extracts of whole cells and cell fractions. The method employs chemically mild conditions; single-strand scissions are not introduced by interaction with the dye, nor is there any permanent rearrangement of the DNA structure. All the steps of the separation

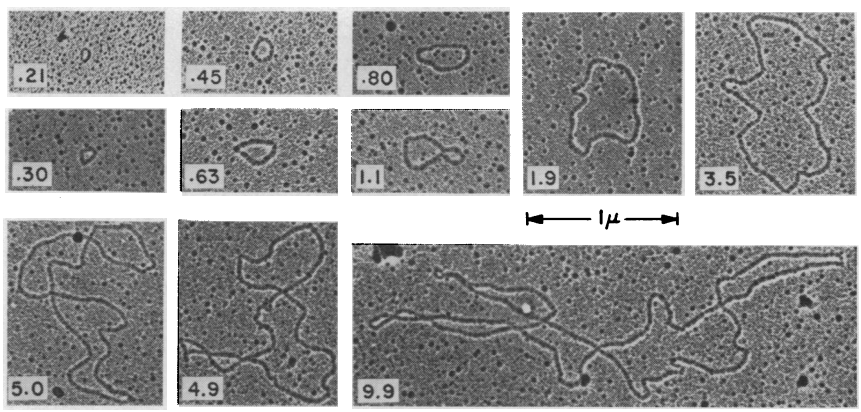


FIG. 6.—Electron micrographs of circular DNA from HeLa cells. Fractions 33–36 from the dense band in Fig. 5*c* were pooled and used in the specimen preparations. The top photographs present selected molecules of the small size range. The number in each insert gives the length in microns of the molecule. The first two molecules in the second row are of mitochondrial size; the third is twice the mitochondrial length.

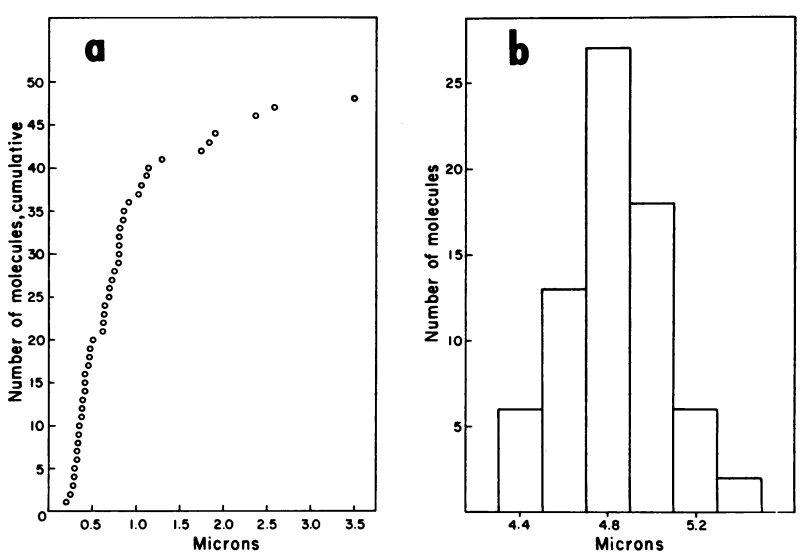


FIG. 7.—Frequency distribution of lengths of circular DNA isolated from the HeLa cell band referred to in the legend for Fig. 6. (*a*) A cumulative frequency distribution of lengths of molecules in the submitochondrial size class. (*b*) A histogram of the distribution of lengths of molecules in the mitochondrial size class.

are carried out at room temperature, or below, and at neutral pH. In the experiments performed so far, the maximum amount of DNA introduced in a single tube was 125 μg of polyoma DNA.

It has become clear, in the course of this initial study of the closed circular DNA in malignant human cells, that a portion of the mitochondrial DNA in HeLa cells is in the form of closed duplexes. These circular molecules are similar in form, size, and homogeneity to the mitochondrial DNA's from invertebrate and vertebrate species previously investigated.[3-5, 16, 17]

The heterogeneous group of small molecules, 0.2–3.5 microns, are to be compared with the size range, 0.5–16.8 microns, reported by Hotta and Bassel[18] to be present in preparations of unfractionated boar sperm DNA. The conclusion that the small circles, seen in our electron micrographs, represent DNA molecules rests on three observations. (1) The small circles were found at the same level in a CsCl-dye gradient as were the closed circular mitochondrial DNA molecules. (2) The grain pattern of the shadowed metal on the small circles viewed at a magnification of 1×10^5 was indistinguishable from the pattern on mitochondrial DNA molecules in the same field. (3) The small circles were seen only very infrequently in DNA preparations from isolated HeLa cell mitochondria. It is thus unlikely that the small circles arise from an artifact in the electron-microscope procedure. A more definitive characterization of these small DNA molecules, which can code for only 200–3500 amino acid residues, requires the preparation of larger quantities of material. The frequency of occurrence of the small molecules in the SDS extracts we have studied does not necessarily represent the frequency of occurrence in the HeLa cell. It is emphasized here that the dye-buoyant method segregates only *closed* circular duplexes. Molecules that contain even one single-strand scission, for whatever reason, find their way to the less dense band and, in whole cell extracts, intermingle with the large excess of linear DNA.

The larger-size circles, which were also seen at a frequency of about 10 per cent relative to mitochondrial DNA in the dense band, are clearly multiples of the mitochondrial length. The mean lengths from measurements of 43 double-length circles and a smaller number of larger multiples were 9.56 ± 0.42, 14.1 ± 0.4, and 19.8 ± 1.1 microns. We have so far not found a fully extended large circle. Nor have we found, in the several large circles which contain infrequent crossovers, any one which could not have arisen from a pairing of two or more mitochondrial molecules. An investigation of the significance of these multiple-size circles in HeLa DNA is in progress. Nass[17] has reported measurements of multiple lengths of mitochondrial molecules liberated from mitochondria by osmotic shock during specimen preparation. She attributed the multiples to the superposition of DNA molecules which originated from single mitochondria.

Summary.—A buoyant-density method for the isolation and detection of closed circular DNA is described. The method is based on the reduced binding of the intercalating dye, ethidium bromide, by closed circular DNA. In an application of this method we have found that HeLa cells contain, in addition to closed circular mitochondrial DNA of mean length, 4.81 microns, a heterogeneous group of smaller DNA molecules which vary in size from 0.2 to 3.5 microns and a paucidisperse group of multiples of the mitochondrial length.

Note added in proof: That double-length closed mitochondrial DNA molecules do occur has been shown by D. Clayton in this laboratory with preparations from leucocytes obtained from the blood of a donor with chronic granulocytic leukemia. Circular DNA molecules free of cross-overs and of twice the mitochondrial length were observed in electron micrographs.

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[1] Dulbecco, R., and M. Vogt, these PROCEEDINGS, **50**, 236 (1963).

[2] Weil, R., and J. Vinograd, these PROCEEDINGS, **50**, 730 (1963).

[3] Borst, P., and G. J. C. M. Ruttenburg, *Biochim. Biophys. Acta*, **114**, 645 (1965).

[4] Kroon, A. M., P. Borst, E. F. J. Van Bruggen, and G. J. C. M. Ruttenburg, these PROCEEDINGS, **56**, 1836 (1966).

[5] Pikó, L., A. Tyler, and J. Vinograd, *Biol. Bull.*, in press.

[6] Crawford, L. V., and P. H. Black, *Virology*, **24**, 388 (1964).

[7] Crawford, L. V., *J. Mol. Biol.*, **8**, 489 (1964).

[8] *Ibid.*, **13**, 362 (1965).

[9] Burton, A., and R. L. Sinsheimer, *J. Mol. Biol.*, **14**, 3276 (1965).

[10] Kleinschmidt, A. K., A. Burton, and R. L. Sinsheimer, *Science*, **142**, 1961 (1963).

[11] Young, E. T., II, and R. L. Sinsheimer, *J. Mol. Biol.*, **10**, 562 (1964).

[12] Bode, V. C., and A. D. Kaiser, *J. Mol. Biol.*, **14**, 399 (1965).

[13] Play, D. S., A. Preuss, and P. H. Hofschneider, *J. Mol. Biol.*, **21**, 485 (1966).

[14] Rhoades, M., and C. A. Thomas, private communication.

[15] Roth, T., and D. Helinski, private communication.

[16] David, I. B., these PROCEEDINGS, **56**, 269 (1966).

[17] Nass, M. M. K., these PROCEEDINGS, **56**, 1215 (1966).

[18] Hotta, Y., and A. Bassel, these PROCEEDINGS, **53**, 356 (1965).

[19] Vinograd, J., J. Lebowitz, R. Radloff, R. Watson, and P. Laipis, these PROCEEDINGS, **53**, 1104 (1965).

[20] Vinograd, J., and J. Lebowitz, *J. Gen. Physiol.*, **49**, 103 (1966).

[21] Vinograd, J., R. Bruner, R. Kent, and J. Weigle, these PROCEEDINGS, **49**, 902 (1963).

[22] M. Gellert, these PROCEEDINGS, **57**, 148 (1967).

[23] Bauer, W. R., and J. Vinograd, in preparation.

[24] Crawford, L. V., and M. J. Waring, *J. Mol. Biol.*, in press.

[25] Kersten, W., H. Kersten, and W. Szybalski, *Biochemistry*, **5**, 236 (1966).

[26] Waring, M. J., *Biochim. Biophys. Acta*, **114**, 234 (1966).

[27] Le Pecq, J., Dissertation, University of Paris (1965).

[28] Hirt, B., these PROCEEDINGS, **55**, 997 (1966).

[29] Kleinschmidt, A. K., and R. K. Zahn, *Z. Naturforsch.*, **14b**, 770 (1959).

[30] LePecq, J., and C. Paoletti, *Anal. Biochem.*, **17**, 100 (1966).

[31] Young, E. T., II, and R. L. Sinsheimer, private communication.