finding of altered base ratios after learning, and evidence from invertebrates) is not included here. While it is still conceivable that a hybrid molecule, consisting partly of a "protected" form of polynucleotide, might correspond to the discovered list of properties, this seems to be an unparsimonious assumption to make without further evidence, and it also seems questionable whether such a molecule could contain the required quantity of information in the small number of nucleotide groups possible within the molecular weight constraints. In any event, further work on the properties and subcellular localization of the molecule seems to be required in order to obtain a definitive answer to this question, and to permit a clear choice between the alternative theories of synaptic modification and genetic induction as the underlying mechanism for the transfer effect.

* This work was supported by U.S. Office of Naval Research contract Nonr 401(40) and National Science Foundation contract GK-250.

[1] Rosenblatt, F., J. T. Farrow, and S. Rhine, these PROCEEDINGS, **55**, 548 (1966).

[2] Siegel, S., *Nonparametric Statistics* (New York; McGraw-Hill, 1956).

---

*ISOLATION OF THE DNA OF THE E. COLI CHROMOSOME IN ONE PIECE**

BY C. I. DAVERN

COLD SPRING HARBOR LABORATORY OF QUANTITATIVE BIOLOGY, COLD SPRING HARBOR, NEW YORK

Communicated by A. D. Hershey, February 10, 1966

The autoradiographs of H^3-labeled chromosomes of *Escherichia coli* prepared by Cairns,[1] and the explanations of the chromosome's growing mechanism by Bonhoeffer and Gierer[2] and Nagata,[3] are consistent with the notion that the DNA of the replicating chromosome is a single molecule with a single growing point. Such a molecule would have a molecular weight of $2.3–4.6 \times 10^9$ daltons,[1] depending on its stage of replication. Attempts to isolate the DNA of the *E. coli* chromosome in one piece, by methods designed to minimize shear and nuclease action, have so far failed,[4, 5] although very large DNA molecules (4×10^8 daltons), corresponding roughly to half the nuclear DNA content, have been isolated from *Hemophilus influenzae* by Berns and Thomas.[5]

Such attempts to relate the isolated DNA molecule to the molecular organization of the bacterial chromosome are indirect, for they rely on comparison of an estimate of the molecular weight of the isolated molecule with an estimate of the chromosome DNA content. This paper describes a more direct method for assessing the relation between the isolated DNA molecule and the DNA of the chromosome. The method was applied to DNA extracted from *E. coli* and banded in a CsCl density gradient by means of a technique designed to avoid shear stress and minimize nuclease action. It showed that the DNA of the *E. coli* chromosome can be isolated in one piece.

The assessment is based on CsCl equilibrium density gradient analysis[6] of DNA isolated from cells that have incorporated 5-bromouracil into their DNA for a portion of the chromosome replication cycle. If each chromosome has all its DNA in

one molecule, then those molecules that were replicating during the period of labeling with 5-bromouracil should be partly hybrid and, if isolated without fragmentation, should be detectably denser than normal.

The sensitivity of the method for detecting fragmentation can be gauged from the following arguments. If the DNA in the chromosome replicates by means of a single growing point, and if about one quarter to one half of each replicating molecule has become hybrid during the labeling period, then the breakage of such molecules into two fragments will produce some fragments that are hybrid in density.

Two innovations were employed in the release of DNA. First, to avoid unnecessary shear, spheroplasts were lysed on top of the CsCl solution in the centrifuge tube. Second, sodium dodecyl sarcosinate was used to promote lysis. Unlike the commonly used detergent, sodium dodecyl sulfate, the sarcosinate is soluble in concentrated CsCl.

Material and Methods.—Bacterial cultures: Escherichia coli strain 15 TAU-bar,[7] which requires thymine, uracil, arginine, proline, tryptophan, and methionine, was grown in glucose-ammonium medium[8] supplemented with 2.5 μg/ml each of L-arginine, L-proline, L-tryptophan, and L-methionine, 10 μg/ml uracil, and 0.02% Difco Bacto-peptone. Although not essential for growth, the peptone supplement was essential for renewal of growth of this strain after filtration on Millipore filters. The bacteria were grown with aeration at 37°C.

C^{14}-labeled DNA: Bacteria were grown for six generations to saturation in the presence of 2 μg/ml C^{14}-thymidine (sp. act. 30 mc/mM), washed, and suspended in 0.01 *M* ethylenediaminetetracetate (EDTA), 0.01 tris(hydroxymethyl) aminomethane (tris buffer), pH 8.5. The cells were then lysed by the sequential addition of 40 μg/ml egg white lysozyme (Worthington Biochemical Corp., Freehold, N.J.), chloroform, and 0.5% sodium dodecyl sarcosinate (Sarkosyl N.L.-97 from Geigy Industrial Chemicals, Ardsley, N.Y.). The cleared lysate, containing approximately 6 μg/ml of DNA, was thoroughly shaken to ensure fragmentation of the DNA.

H^3-thymidine labeling of 15 TAU-bar: The cells were grown in 0.5 ml of the supplemented glucose-ammonium medium containing 4 μg/ml H^3-thymidine (sp. act. 3.35 c/mM) for 3–4 generations to 10^8 ml, whereupon they were filtered and washed on an HA Millipore filter. Because of the small quantity of labeled cells being filtered, the filters had to be pretreated with unlabeled cells to ensure good recoveries.

Density labeling of the chromosome with 5-bromouracil: The washed H^3-thymidine-labeled cells were suspended in growth medium containing 40 μg/ml 5-bromouracil in place of thymidine, and then incubated with aeration at 37°C. At the end of a labeling period, the culture was filtered.

Spheroplast formation:[9] The cells from the filter were suspended in 0.3 ml spheroplasting medium consisting of 20% w/w sucrose and 1 mg/ml egg white lysozyme in 0.01 *M* EDTA, 0.01 *M* tris buffer, pH 8.5, and converted to spheroplasts by incubation at 37°C for 30 min.

Lysis of spheroplasts: The following solutions were layered successively into a centrifuge tube containing 0.02 ml C^{14}-DNA: (1) 4 ml 60% w/w CsCl in 0.01 *M* phosphate buffer, pH 6.4; (2) 0.5 ml of 40% w/w glycerol in 0.01 *M* EDTA, 0.01 *M* tris buffer, pH 8.5, containing 0.5% sodium dodecyl sarcosinate and 100 μg/ml pronase (Calbiochem, Los Angeles, Calif.); and (3) 0.5 ml 0.01 *M* EDTA, 0.01 *M* tris buffer pH 8.5, containing 100 μg/ml pronase. Immediately after preparation of the centrifuge tube in this manner, 0.02 ml of the spheroplast suspension (containing 2–4 $\times$ 10^7 spheroplasts/ml) was carefully applied at the junction between the glycerol layer and the top buffer layer. The tube was then covered with parafilm and left undisturbed for at least 16 hr before being centrifuged. During this period, detergent and pronase (which is active in the presence of the detergent) diffused into the spheroplast layer and lysed the spheroplasts. Meanwhile, CsCl diffused to form a gradient similar to the one achieved by centrifugation.

Equilibrium density gradient centrifugation: The samples were centrifuged at 35,000 rpm for at least 50 hr at 15–20°C in the SW39 rotor of the Spinco model L ultracentrifuge. At the end of the run the meniscus from each tube was sampled by dabbing with a disk of Whatman no. 1 filter paper, and the remainder of the solution was collected in 5-drop fractions directly on 1-in. squares of Whatman no. 1 filter paper in vials. The empty tubes were cut transversely into three pieces,

and each piece was inserted into a separate vial. The samples were dried, and the radioactivity was measured in a scintillation spectrometer.

Results.—Log-phase bacteria, uniformly labeled with H^3-thymidine, were transferred to warm medium containing 5-bromouracil. Samples were taken immediately before, 40 min after, and 87 min after transfer, and the cells from each sample converted to spheroplasts. Three centrifuge tubes, containing the buffer-glycerol lysing layers floating on concentrated CsCl, were each carefully loaded with a 0.02-ml aliquot of spheroplasts at the buffer-glycerol boundary. Tubes 1 and 2 received the spheroplasts from the 40-min sample. Tube 3 received a mixture of spheroplasts from the 0-min and 87-min samples. The tubes were left undisturbed for 16 hr, then the contents of tube 2 were subjected to shear by five passages through a Pasteur pipet. All tubes were then centrifuged for 52 hr at 35,000 rpm.

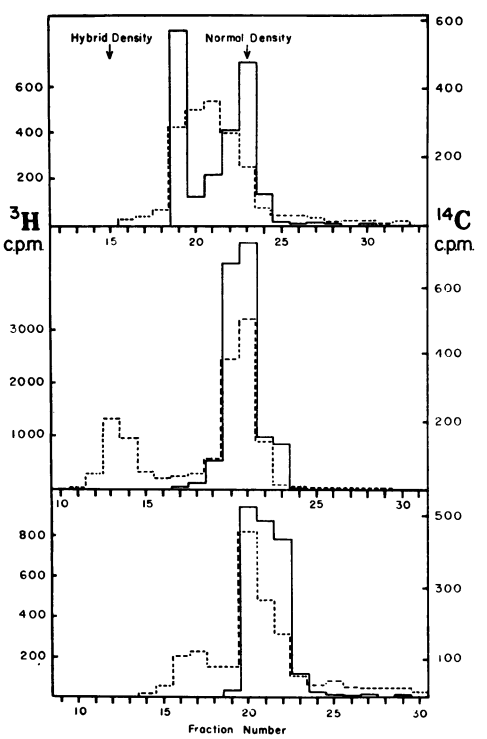

FIG. 1.—Density distributions of H^3-DNA (- - - - -) released from spheroplasts and C^{14}-DNA (——) used as a density marker. The spheroplasts were prepared from log-phase cells, uniformly labeled with H^3-thymidine, that had been incorporating 5-bromouracil for the times indicated. *Top:* (tube 1) H^3-DNA from cells labeled with 5-bromouracil for 40 min. *Middle:* (tube 2) H^3-DNA from same sample as tube 1, subjected to shear immediately before centrifugation. *Bottom:* (tube 3) H^3-DNA from a mixture of cells not labeled with 5-bromouracil and cells so labeled for 87 min.

The density distribution of the H^3-DNA derived from the lysed spheroplasts is shown in Figure 1. In the middle frame of the figure, a calibration of the density gradient is provided by the distribution of label from the deliberately sheared sample. Here the left-hand peak marks the position of hybrid DNA, and the other peak marks that of DNA of normal density. This profile also shows that 27 per cent of the spheroplast DNA present at the beginning of transfer had replicated to become hybrid during the 40 min of labeling with 5-bromouracil.

Density distribution of unsheared DNA: DNA extracted from the 40-min sample of spheroplasts formed a single broad band ranging from normal to almost hybrid density. No DNA was detectable at the position corresponding to the density of hybrid DNA (Fig. 1, *top*). DNA extracted from the mixture of spheroplasts from the 0-min and 87-min samples formed two bands, one corresponding to DNA of normal density, and the other to DNA of a density intermediate between hybrid and normal. Again no DNA of hybrid density was detectable.

Trapping of DNA by spheroplast DNA band: The anomalous distribution in tube 1 of the C^{14}-DNA used as a density marker (Fig. 1, *top*) indicates that the very small amount of unsheared DNA (about 0.002 μg) in the spheroplast

TABLE 1

RECOVERIES, AFTER CENTRIFUGATION, OF H^3-DNA FROM LYSED SPHEROPLASTS AND C^{14}-DNA USED AS A DENSITY MARKER

	Tube 1*		Tube 2*		Tube 3*	
Location of label	H^3 (%)	C^{14} (%)	H^3 (%)	C^{14} (%)	H^3 (%)	C^{14} (%)
Banded in gradient	23	98	98	98	17	97
At meniscus	46	0	1	0	69	0
Adsorbed to centrifuge-tube walls	31	2	1	2	14	3

* Loaded with spheroplasts as described in legend of Fig. 1.

DNA band can impede the free sedimentation of other DNA through it. The absence of such trapping by the smaller amount of unsheared DNA in tube 3 (Fig. 1, *bottom*) suggests that trapping depends on concentration. Results of an experiment in which a sample three times smaller than those of Figure 1 was analyzed confirm that suggestion (Fig. 2).

DNA complex of low density: Although extraction as described yielded some DNA banding in the gradient at a density expected for pure DNA, much of the DNA from the lysed spheroplasts was recovered from the meniscus and the tube wall (Table 1). Most of the DNA that ended up on the tube walls was in fact derived from DNA left behind at the meniscus after its rather inefficient sampling. Shearing of this complex permitted nearly complete recovery of DNA of normal density.

The buoyant density of the complex was measured by using 56 per cent CsCl in the CsCl layer. Under these conditions, the DNA complex banded one third of the distance from the meniscus to the tube bottom, corresponding to a density of about 1.55 gm/ml.

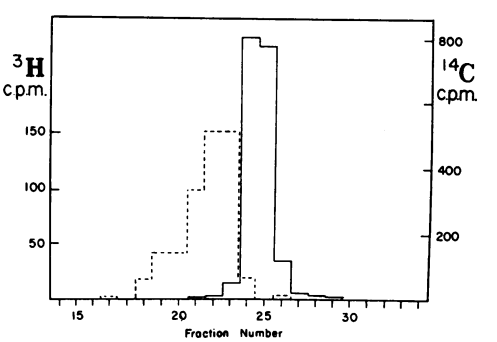

FIG. 2.—Density distribution of H^3-DNA (- - - - -) released from spheroplasts and C^{14}-DNA (——) used as a density marker. The spheroplasts were prepared from log-phase cells, uniformly labeled with H^3-thymidine, that had been incorporating 5-bromouracil for 60 min.

Discussion.—The results show that detergent and pronase release only part of the DNA from spheroplasts in the free state. Most of it remains bound to a low-density material in an extended form that is sensitive to shear.

The density distribution of the free DNA is consistent with that expected for a population of molecules heterogeneous in size, each of which has been partially replicated with 5-bromouracil as a precursor, and all or most of which have been isolated without fragmentation.

Very low concentrations of this DNA in the band can trap fragmented DNA also present in the preparation subjected to density gradient centrifugation. This phenomenon is another indication of the large size of the unsheared DNA. However, the same phenomenon may conceal the presence of any fragments of hybrid density generated by the extraction procedure. Even in the experiment shown in Figure 2, where there is no evidence of trapping of severely fragmented DNA, no guarantee can be given that there would be no trapping of the much larger fragments that

might arise during extraction. On the other hand, spheroplast DNA of two density species deliberately mixed can be separated, at least in part (Fig. 1, *bottom*). This result makes it unlikely that any considerable fragmentation would be missed in these experiments. Thus, in spite of poor recoveries and banding anomalies, the data allow one to conclude that the entire DNA of the chromosome can be isolated as a single molecule, provided, of course, that the chromosome replicates by way of a single growing point. Massie and Zimm[4] concluded, on the contrary, that the chromosome of *E. coli* consists of some eight or so subunits joined together by pronase-susceptible linkers.[10]

The extraction procedure described in this paper yields most of the DNA from spheroplasts in a complex of low density. Virtually all of the DNA in the complex is sensitive to shear and must be present in extended form. The complex could originate by casual entanglement of the emerging DNA molecule with spheroplast debris, or could reflect the persistence of a chromosome-membrane association.[11-13] Whatever the nature of the complex, DNA associated with it must, like the free DNA, be unfragmented.

Owing to the banding anomalies already referred to, the method used here for assessing fragmentation becomes insensitive when the amount of DNA in the band exceeds 0.001 μg. The use of 5-bromouracil as a density label imposes further limitations because the analogue depresses the replication of DNA and may disturb its regulation.[14, 15]

Cairns' autoradiographs show that the replicating *E. coli* chromosome has the configuration of a circularized Y with fused ends.[1] Fragmentation of such a structure requires at least two or three breaks. Thus, a test for fragmentation cannot be used to assess the intactness of the chromosome, a limitation it shares with other methods.

Summary.—A method is described for extracting the DNA from *E. coli* by lysing spheroplasts on top of a CsCl solution by the combined action of pronase and sodium dodecyl sarcosinate. Density gradient analysis of DNA isolated by this method from cells that have incorporated 5-bromouracil into their DNA for about one third of a replication cycle reveals that the entire DNA complement of the chromosome can be isolated as one molecule.

The author wishes to thank Dr. H. J. F. Cairns for persuading him to tackle this project, and to acknowledge the skilled assistance of Miss Paula de Lucia.

* This work was supported by the National Science Foundation.

[1] Cairns, H. J. F., *J. Mol. Biol.*, **6**, 208 (1963); Cairns, H. J. F., in *Synthesis and Structure of Macromolecules*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 28 (1963), p. 43.

[2] Bonhoeffer, F., and A. Gierer, *J. Mol. Biol.*, **7**, 534 (1963).

[3] Nagata, T., these PROCEEDINGS, **49**, 551 (1963).

[4] Massie, H. I., and B. H. Zimm, these PROCEEDINGS, **54**, 1636 (1965).

[5] Berns, K. I., and C. A. Thomas, *J. Mol. Biol.*, **11**, 476 (1965).

[6] Meselson, M., F. W. Stahl, and J. Vinograd, these PROCEEDINGS, **43**, 581 (1957).

[7] Hanawalt, P. C., *Nature*, **198**, 286 (1963).

[8] Hershey, A. D., *Virology*, **1**, 108 (1955).

[9] Spheroplasting procedure modified from methods suggested by Neu, H. C., and L. A. Heppel, *J. Biol. Chem.*, **239**, 3893 (1964); and Repaske, R., *Biochim. Biophys. Acta*, **30**, 225 (1958).

[10] Freese, E., in *Exchange of Genetic Material: Mechanisms and Consequences*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 23 (1958), p. 13.

[11] Goldstein, A., and B. J. Brown, *Biochim. Biophys. Acta*, **53**, 19 (1961).
[12] Ryter, A., and F. Jacob, *Ann. Inst. Pasteur*, **107**, 384 (1964).
[13] Ganesan, A. T., and J. Lederberg, *Biochem. Biophys. Res. Commun.*, **18**, 824 (1965).
[14] Lark, K. G., T. Repko, and E. J. Hoffman, *Biochim. Biophys. Acta*, **76**, 9 (1963).
[15] Pettijohn, D., and P. Hanawalt, *J. Mol. Biol.*, **9**, 395 (1964).

*ALKYLATION OF A BRAIN TRANSPORT ADENOSINETRIPHOSPHATASE AT THE CARDIOTONIC STEROID SITE BY STROPHANTHIDIN-3-HALOACETATES**

BY LOWELL E. HOKIN,† MICHAEL MOKOTOFF,‡ AND S. MORRIS KUPCHAN

DEPARTMENTS OF PHYSIOLOGICAL CHEMISTRY AND PHARMACEUTICAL CHEMISTRY, UNIVERSITY OF WISCONSIN, MADISON

Communicated by Henry Lardy, February 1, 1966

Our understanding of the molecular mechanisms involved in membrane transport has been hampered by our inability to isolate the macromolecules concerned. A promising development in the field of Na^+ and K^+ transport was the demonstration of an adenosinetriphosphatase (ATPase) activity closely related to Na^+ and K^+ transport (see review by Skou[1]). This enzyme is dependent on the simultaneous presence of Na^+ and K^+ and is inhibited by cardiotonic steroids, which rather specifically inhibit Na^+ and K^+ transport.[2, 3] The ATPase is usually isolated in a lipoprotein particulate fraction derived from cell membranes, and all attempts to purify it extensively have failed. One approach to the transport problem is to react the macromolecular transport components with radioactive irreversible inhibitors so as to tag the macromolecular components. Reagents such as diisopropylfluorophosphate (DFP) have been extensively used in labeling the active sites of numerous animal hydrolases (see, for example, Oosterbaan and Cohen[4]). In an earlier study we found that DFP irreversibly inhibited the ($Na^+ + K^+$) activated ATPase (hereafter referred to as transport ATPase) in a kidney membrane preparation;[5] the nontransport ATPase was unaffected. Although the inhibition by DFP and the labeling of the protein by DFP^{32} were reduced by ATP, in further studies with a brain transport ATPase it was not possible to establish conditions which demonstrated to our complete satisfaction a labeling of the transport ATPase distinct from the labeling of other proteins in the crude enzyme preparation;[6] this limited the use of DFP as a specific labeling agent for the transport ATPase.

In recent years the labeling of active sites of enzymes has been accomplished by incubating with substrates containing reactive functional groups which form covalent bonds at the substrate sites.[7–11] These reagents have been termed "active-site-directed irreversible inhibitors."[7] We thought that such a reagent would be more useful than DFP in labeling a specific site on the transport ATPase. Obviously, derivatives of the natural activators, i.e., Na^+ and K^+, could not be prepared. However, the high affinity of cardiotonic steroids for the transport ATPase offered an approach. The preparation of derivatives of strophanthidin was decided upon. This cardiotonic steroid offers several advantages for this kind of work. It is a specific inhibitor of the transport ATPase,[3] it inhibits reversibly,[5, 6]