[17] Bressler, R., and S. J. Wakil, *J. Biol. Chem.*, **236**, 1643 (1961).
[18] Hsu, R. Y., G. Wasson, and J. W. Porter, *Federation Proc.*, **24**, 291 (1965).
[19] Martin, D. B., and P. R. Vagelos, in *Handbook of Physiology—Section 5-Adipose Tissue*, ed. A. E. Renold and G. F. Cahill, Jr. (Baltimore, Md.: Waverly Press, Inc., 1965), p. 211.
[20] Trams, E. G., and R. O. Brady, *J. Am. Chem. Soc.*, **82**, 2972 (1960).
[21] Simon, E. J., and D. Shemin, *J. Am. Chem. Soc.*, **75**, 2520 (1953).
[22] Moffatt, J. G., and H. G. Khorana, *J. Am. Chem. Soc.*, **83**, 663 (1961).
[23] Novelli, G. D., in *Methods in Enzymology*, ed. S. P. Colowick and N. O. Kaplan (New York: Academic Press, Inc., 1955), vol. 2, p. 667.
[24] Vagelos, P. R., unpublished observation.
[25] Martin, D. B., M. G. Horning, and P. R. Vagelos, *J. Biol. Chem.*, **236**, 663 (1961).
[26] Stadtman, E. R., in *Methods in Enzymology*, ed. S. P. Colowick and N. O. Kaplan (New York and London: Academic Press, Inc., 1957), vol. 4, p. 228.
[27] Bakerman, H., M. Romine, J. A. Schricker, S. M. Takahashi, and O. Mickelsen, *Agr. Food Chem.*, **4**, 956 (1956).
[28] Warburg, O., and W. Christian, *Biochem. Z.*, **310**, 384 (1941–1942).
[29] Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
[30] Ellman, G. L., *Arch. Biochem. Biophys.*, **82**, 70 (1959).
[31] Bray, G. A., *Anal. Biochem.*, **1**, 279 (1960).
[32] Daft, F. S., E. G. McDaniel, L. G. Herman, M. K. Romine, and J. R. Hegner, *Federation Proc.*, **22**, 129 (1963).
[33] Ames, B. N., and D. T. Dubin, *J. Biol. Chem.*, **235**, 769 (1960).

---

*EARLY INTRACELLULAR EVENTS IN THE REPLICATION OF T4 PHAGE DNA, I. COMPLEX FORMATION OF REPLICATIVE DNA**

BY ANDRZEJ W. KOZINSKI AND T. H. LIN†

THE WISTAR INSTITUTE, PHILADELPHIA, PENNSYLVANIA

Communicated by Thomas F. Anderson, May 3, 1965

When *E. coli* B, infected with P^{32}-labeled bacteriophage T4, is disrupted with duponol or lysozyme, and the resulting lysate analyzed in a cesium chloride density gradient, a large fraction of the parental label forms a band in the gradient, while after draining CsCl solution, the residual radioactivity associated with the floating pellet can be extracted from the tube with hot alkali. If the duponol or lysozyme lysate is extracted with phenol, approximately one half of the P^{32} becomes adsorbed at the interphase between the water and the phenol. Repeated phenol extraction does not release P^{32} from the interphase pellet.[5]

This and the following paper will analyze the novel properties of T4 DNA during early stages of its replication.

Materials and Methods.—The bacterial strain used in these experiments was *E. coli* B. The bacteriophage was T_4BO_r.[1] Light, heavy, radioactive, and starvation synthetic TCG media along with the methods for purification of bacteriophage and the sucrose and CsCl density gradient techniques were described before.[2–4]

Intracellular DNA extraction procedures: (*I*) Duponol-phenol extraction: Infected bacteria were sedimented, washed once with EDTA (0.15 *M* NaCl-0.015 *M* EDTA, pH 8.0), and resuspended to a concentration of 3–6 $\times$ 10^8 bacteria per milli-

liter. This is the critical concentration as the efficiency of extraction E (see description below) becomes erratic at higher concentrations of bacteria. The suspension was lysed at 37°C for 10 min with purified duponol at a final concentration of 0.5 per cent. The clear lysate was chilled and extracted with 1–2 vol of phenol at room temperature, revolving in a tube held at an angle and at 45–60 rpm for 45 min. The mixture was centrifuged and the phenol layer removed. The excess of phenol was removed from the aqueous phase by gentle washing with ether (3 ×) or by dialysis. A second phenol extraction did not yield greater recovery of injected DNA. (*II*) Sonication-phenol extraction: A bacterial suspension at 3–6 × 10^8 in EDTA was lysed in a Raytheon sonicator adjusted to maximum output for 5 min. The lysate was extracted with phenol in a manner identical to that described above. (*III*) Duponol-pronase-phenol: A duponol lysate was supplemented with pronase at a concentration of 1 mg pronase per milliliter of lysate and incubated at 37°C for 5–7 hr. Enzymatic digestion was followed by phenol extraction as described in procedure *I*.

In all of these methods an adequate amount of H^3 reference DNA (usually 3 × the counts of P^{32}) was added to the EDTA suspension of bacteria. A sample of the mixture was transferred to a fiber glass filter pad prior to extraction. After extraction a sample was taken and transferred to another fiber glass filter pad. The samples were dried, overlaid with scintillation liquid, and assayed in a liquid scintillation counter adjusted for double channel measurements. The efficiency (E) of extraction of intracellular P^{32} was estimated by comparing the relative activity of both isotopes:

$$E = \frac{(H^3/P^{32}) \text{ prior to extraction}}{(H^3/P^{32}) \text{ after extraction}}.$$

Results.—(A) *Preferential incorporation of replicative DNA into a complex non-extractable with phenol:* Heavy, cold 5-BU-labeled bacteria grown for two generations in a medium supplemented with 5-BU were infected with radioactive T4 bacteriophage at a m.o.i. of 1.0. (The same results were obtained when bacteria were infected at higher multiplicities (3–8) of infection.) At various time intervals after infection, samples of the bacterial suspension were chilled and centrifuged. The pellet was washed once with an EDTA solution (0.15 *M* NaCl and 0.015 *M* EDTA pH 8) and resuspended at a final concentration of 3–6 × 10^8 bacteria per milliliter; H^3 light reference DNA was added. The bacterial suspension was divided into two parts: one part was lysed with duponol and the other subjected to sonication. A fraction of the lysed bacteria was extracted with phenol (procedures *I* and *II*). Part of the phenol-extracted DNA and duponol lysates were each analyzed in a CsCl density gradient centrifugation. Fractions were collected on fiber glass filter pads, dried, and counted in a liquid scintillation spectrometer (Fig. 1). The other parts of the extracts were used for the comparison of the efficiency of extraction of the injected parental P^{32}. The results are shown in Table 1.

Both Figure 1 and Table 1 (*A* and *B*) indicate unambiguously that during phenol extraction of the duponol-lysed bacteria the replicative moiety of DNA is preferentially incorporated into the interphase pellet and therefore removed from the resulting extract. Lysis of infected bacteria by sonication prior to phenol extraction

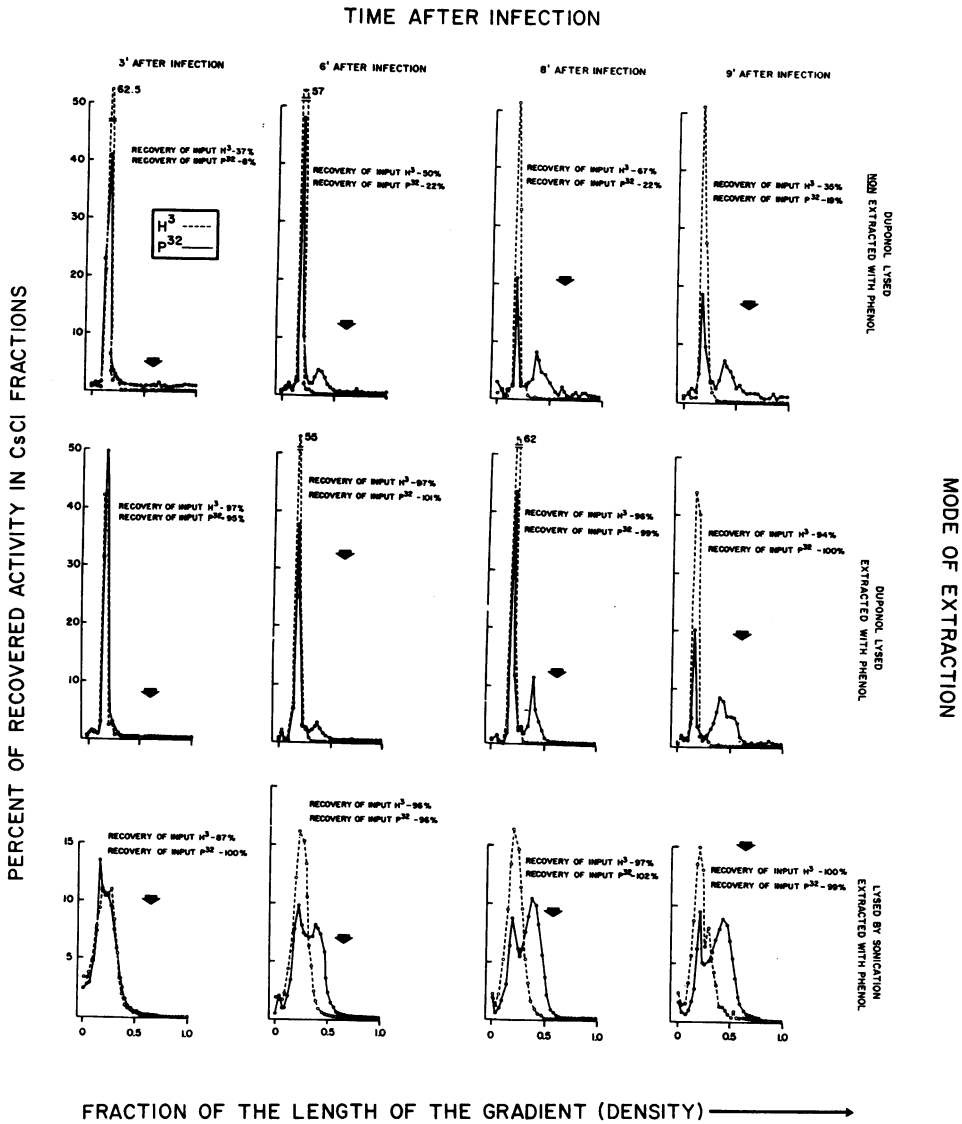

FIG. 1.—CsCl density gradient fractionation of intracellular DNA. The effect of the type of extraction on the replicative pattern. Conditions of centrifugation: 33,000 rpm, 72 hr, 22°C; 9.1 *M* CsCl in CS. The thick arrows represent the *heavy* DNA location.

releases DNA from the complex and allows quantitative recovery of P^{32}-labeled DNA in the aqueous phase.

(B) *Nature of the DNA-protein complex and conditions necessary for its formation:* The results described in the preceding section indicate that replicative DNA is being preferentially incorporated either to the pellet floating on the top of CsCl gradient when crude duponol lysates are analyzed, or absorbs to the interphase pellet during phenol extraction. In the former case, a large fraction of reference H^3-DNA is also incorporated to the floating pellet. These erratic distribution patterns are, as indicated by the proper control, concentration-dependent, which is in

TABLE 1

Type of Sample: Type of bacteria, phage, and conditions of incubation	Time after infection (min)	Type of Extraction and Efficiency (*E*): Duponol-phenol	Sonication-phenol	Duponol-pronase-phenol
A. Heavy, nonstarved bacteria infected; m.o.i. = 1.0 with P^{32} light phage; samples used for CsCl centrifugation shown in Fig. 1	3	1.01	1.00	—
	6	0.47	1.02	—
	8	0.45	0.95	—
	9	0.45	1.00	—
B. Light, starved (40 min) bacteria infected; m.o.i. = 3.0 with heavy P^{32} phage viable; energy sources added after 5 min = time 0	3	0.95	0.98	—
	4	0.87	1.00	0.93
	5	0.58	1.00	0.95
	6	0.59	1.02	0.89
	9	0.61	1.01	0.93
	10	0.68	1.00	0.90
	15	1.05	1.01	1.01
C. Same suspension of infected bacteria as in *B*, but NO energy added; time 0 = 5 min after infection	0	0.97	1.00	—
	3	1.10	1.02	—
	6	1.04	1.05	—
	9	0.97	1.01	—
D. Same suspension as in *B;* CM (100 μg/ml) added at the moment of infection; energy sources added at 5 min = time 0	0	1.00	1.08	0.97
	9	0.70	1.00	0.95
	20	0.67	1.01	0.98
	50	0.61	0.98	0.94
E. Same suspension as in *B;* CM added 6 min after addition of energy	20	0.59	1.01	1.01
	50	0.68	1.02	0.99
F. Same suspension as in *B;* infected with light P^{32}, UV-inactivated (100 hits) T4 phage; m.o.i. = 3.0; energy added 5 min after infection	3	0.94	1.02	—
	9	0.69	0.99	—

definite contrast to the rather reproducible range of partition encountered during phenol extraction. Since these observations can be explained as association of replicative DNA with protein, the following experiments were designed to analyze this phenomenon more extensively. All of the experiments were performed simultaneously using the same batch of bacteria which was subdivided to permit introduction of variables such as starvation, inhibition of protein synthesis at different times, and infection with UV-inactivated bacteriophage.

(a) *Effect of starvation on the formation of the nonextractable complex of injected parental DNA:* A bacterial suspension of a concentration of 3×10^8 bacteria per milliliter was transferred from a regular growth medium to a starvation medium and aerated for 45 min at 37°C. This was followed by infection with a heavy P^{32}-labeled bacteriophage (m.o.i. = 3.0). To a part of the suspension, energy sources were added 5 min later. At different times after infection, samples were withdrawn from both the starvation and complete media, chilled, lysed either by duponol or by sonication, and then subjected to phenol extraction. The results (Table 1, *B* and *C*) indicate that injected DNA does not form a complex with protein in starved bacteria.

(b) *Effect of proteolytic enzymes on the efficiency of extraction of intracellular phage DNA:* A fraction of the infected bacteria in complete medium, described in the previous section, was lysed with duponol and treated with proteolytic enzymes. Pronase was found to be most suitable, since it increased to 100 per cent the efficiency of extraction of the injected phage DNA at the critical times of 6–13 min after infection. (See *Materials and Methods* and Table 1, *B*, *D*, and *E*.) The

effect of proteolytic enzymes confirms the assumption that replicative DNA is associated with protein.

(c) *Effect of the inhibition of protein synthesis on the formation of the DNA-protein complex:* Starved bacteria were infected with heavy P^{32} phage at a m.o.i. of 3.0 and subdivided into two flasks. To one, CM was added immediately. Five minutes after infection, energy sources were added to both flasks. At 6 min after addition of energy sources, CM was added to the second flask. Samples for extraction were withdrawn at 20 and 50 min (Table 1, *D* and *E*). It is apparent that addition of CM, even at the moment of infection (a condition under which no replication of DNA occurs) does not prevent injected DNA from entering the complex.

(d) *Intracellular complex formation of UV-inactivated T4 phage DNA:* Bacteria grown in TCG medium and starved for 40 min were infected at a multiplicity of 3.0 with T4 bacteriophage which had been inactivated with UV (100 hits). Five minutes later, energy sources were added. At different times samples were taken and used for extraction. The results indicate that UV-inactivated DNA also enters the complex. Here again, as in experiments with CM added at early times after infection, part of the injected DNA enters the complex nonextractable with phenol (Table 1, *F*).

Discussion.—The process of T4 bacteriophage reproduction in infected bacteria involves semiconservative replication of parental DNA which is accompanied by molecular exchanges; this results in fragmentation of the parental strands and dispersion among a large fraction of the progeny molecules. The parental contribution to a single progeny molecule is 5–7 per cent and is confined to a single fragment covalently bound to the rest of the progeny strand.[1,2]

Inhibition of protein synthesis 5–7 min after infection allows production of the enzymes responsible for semiconservative replication of DNA but interferes with the production of the enzyme responsible for molecular exchanges—recombinase. Recombination, therefore, is an enzymatic process.[3, 4, 6]

When a bacterial suspension was extracted by the duponol-phenol method, a large fraction of the injected parental P^{32} became associated with the interphase pellet.[5] Nothing has been known about the mechanism of these firm binding nor the molecular milieu in which replication and recombination of T4 occurs. The present data indicate that prior to replication the injected T4 DNA enters into a complex with a protein.

Up to 4 min after infection, no complex formation occurred, as measured by the preferential adsorption of parental P^{32}-labeled DNA to the interphase pellet during phenol extraction. At later times there is a drastic change in the organization of intracellular DNA leading to the formation of a complex which is, for the most part, nonextractable with phenol. After 13 min, closely preceding the withdrawal of DNA from the vegetative pool and the maturation of the bacteriophage, there is an increase in the efficiency of extraction.

Injected parental DNA can be released from this complex either by sonication or by treatment with the proteolytic enzyme—pronase. After either treatment, the recovery after phenol extraction is close to 100 per cent.

A comparison in CsCl of the replicative pattern of the injected DNA, extracted either with the duponol-phenol, sonication-phenol, or duponol-pronase-phenol method, indicates that the replicative DNA is the moiety which is preferentially

absorbed to the nonextractable pellet. The fact that the replicated moiety of DNA can be separated from the complex by proteolytic digestion, and that with the crude duponol lysates, the replicated DNA absorbs to a pellet floating on the surface of the CsCl strongly suggests that the DNA becomes associated with a protein.

This protein exists in the cell prior to infection with bacteriophage, as the addition of CM at the time of infection does not affect complex formation. The complex formation, however, is an energy-requiring process which does not occur in starved bacteria. Sonication or treatment with pronase effected efficient recovery of the replicative DNA, thus providing evidence that all injected parental DNA undergoes semiconservative replication. This finding eliminates the remote possibility of replication of only one strand of DNA.

DNA of UV-inactivated bacteriophage when injected into bacteria also enters the complex. These results are consistent with the complex formation which takes place despite the lack of active DNA replication in the presence of CM.

The possible regulatory role of complex formation in DNA replication should be emphasized.

The dissociation of the complex with proteolytic enzymes and the efficient extraction of intracellular DNA allowed us to isolate, at early times after infection (5–6 min), the parental DNA, which is only partially replicated. These partially replicated molecules (PRM), when separated in CsCl, showed several new properties: circularization, lack of the repair of polynucleotide chain continuity, apparent larger size of the molecules, and possibly some partial separation of strands. Description of these properties will be a topic of the subsequent paper.

Summary.—Upon injection, parental DNA enters a proteinaceous complex in which the replicative moiety of DNA is preferentially located. This complex can be disrupted by sonication or treatment with proteolytic enzymes. With parental phage of good viability, all the injected DNA undergoes semiconservative replication. The complex formation occurs in the presence of CM. The DNA of UV-inactivated phage is also complexed upon injection. Complex formation as a prerequisite for replication and possibly recombination is emphasized.

Abbreviations: DNA, deoxyribonucleic acid; 5-Bu, 5-bromodeoxyuridine; FUDR, 5-fluorodeoxyuridine; "hot," labeled with radioactive isotope; "cold," not labeled with radioactive isotope; "heavy," substituted with heavy density marker 5-BU; "light," not substituted with heavy density marker 5-BU; CM, chloramphenicol; m.o.i., multiplicity of infection; EDTA, disodium ethylenedinitrilotetraacetate; CS, citrate salt buffer; CSF, citrate salt buffer with 1% formaldehyde; TD, thymidine; replicative DNA, moiety of parental DNA which during semiconservative replication acquires a new strand (of a new density in particular experimental system).

* This research was sponsored by National Science Foundation grants GB-1 and GB-3016.

† Senior postdoctoral fellow, National Institutes of Health.

[1] Kozinski, A. W., *Virology*, **13**, 124 (1961).

[2] Kozinski, A. W., and P. B. Kozinski, *Virology*, **20**, 213 (1963).

[3] Kozinski, A. W., P. B. Kozinski, and P. Shannon, these PROCEEDINGS, **50**, 746 (1963).

[4] Kozinski, A. W., and P. B. Kozinski, these PROCEEDINGS, **52**, 211 (1964).

[5] Kozinski, A. W., and M. Bessman, *J. Mol. Biol.*, **3**, 746 (1961).

[6] Mead, C. G., these PROCEEDINGS, **52**, 1482 (1964).