DNA REPLICATION IN ESCHERICHIA COLI MADE PERMEABLE BY TREATMENT WITH HIGH SUCROSE

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ABSTRACT:
Plasmolysed E. coli (pol A$^-$, end$^-$)[1] carry out semiconservative, ATP-dependent incorporation of (α^{32}P) deoxynucleoside monophosphates into DNA. The incorporation is temperature-sensitive in dna B, dna E and dna G temperature sensitive mutants, requires four deoxynucleotides (dNTP's or dNMP's) and Mg^{++}, and is sensitive to inhibition by nalidixic acid or N-ethylmaleimide. Pulse-labeled DNA resembles Okazaki fragments and can be chased into larger material. Cells which have stopped DNA replication in vivo due to chloramphenicol treatment show little incorporation after plasmolysis.

The plasmolysed cells are permeable to added DNase I as shown by degradation of 95% of parental DNA and 90-95% inhibition of synthesis of new DNA. Trypsin or exonuclease III also inhibits deoxynucleotide incorporation about 90%.

Introduction

It has become clear that the synthesis carried out in vitro by DNA polymerases does not resemble DNA replication in vivo. Although the T4-induced DNA polymerase is necessary for the replication of T4 DNA, this may be an indirect requirement and certainly this enzyme is not sufficient to carry out replication. Thus, there are other factors needed, in both bacterial and phage systems, to help polymerases replicate DNA. Some of these have been defined genetically by amber or temperature sensitive mutants defective in DNA synthesis under the non-permissive conditions.

Several permeabilized cell preparations which appear to carry out DNA synthesi have recently been described (3-8). However, toluene-treated cells are not fully permeable to macromolecules in our hands (9) while the cellophane disc system of Schaller et al. is somewhat laborious, especially when used as a routine enzyme assay. We thus sought to devise a simpler system, permeable to macromolecules and capable of carrying on DNA replication at a rapid rate. We have found that plasmolysed cells satisfy these requirements.

Materials and Methods

E. coli D110 (polA$_1$, end$^-$, thy$^-$) is a derivative of W3110 obtained from Dr. R.

[1] PolA$_1$ strains lack DNA polymerase I (1), while end$^-$ strains lack endonuclease I (2

Moses. E. coli 1029 ($polA_1$, end^-, thy^-, dna B ts), E. coli NY73 ($polA_1$, thy^-, dna G3, rif^r, str^r, leu^-, met E) and E. coli BT1026 (dna E ts, $polA_1$, end^-, thy^-) were obtained from Dr. J. Wechsler. Strains of E. coli 1029 and BT1026 were originally isolated by F. Bonhoeffer and co-workers and we gratefully acknowledge their permission to use these mutants prior to publication. E. coli NY73 is derived from E. coli strain PC3, a dna G ts mutant originally isolated by P. Carl (10). DC medium contains, per liter, 10 g of casamino acids, 10 g of glucose, 0.1 g of $MgSO_4$, 5 g of NaCl, 1g of NH_4Cl, 10 mg of yeast extract, 3.5 g of K_2HPO_4 and 1.5 g of KH_2PO_4. TG medium contains 0.5 g of NaCl, 8 g of KCl, 1.1 g of NH_4Cl, 0.2 g of $MgCl_2$, 12.2 g of Tris base, and 0.8 g of pyruvic acid per liter. The pH is adjusted to 7.4 before autoclaving. After autoclaving, to each liter is added 1 ml of 0.16 M Na_2SO_4, 2 ml of 1 M $CaCl_2$, 1 ml of 10^{-4}M $FeCl_2$, 5 ml of 40% glucose and 5 ml of thiamine (2 mg per ml).

Preparation of plasmolysed cells: Cells were routinely grown in DC medium supplemented with 2 μg per ml of (^{3}H)-thymine (20 mCi per mmole). Cells were harvested in log phase by chilling the culture on ice, centrifuging for 5 min at 13,000 x g, and washing the pellet once with 0.02 volume of cold TG medium supplemented with 2 μg per ml of thymine. The pelleted cells were finally suspended by vortexing in 0.04M Tris chloride pH 8.0, 0.010 M ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid and 2 M sucrose at a concentration of about 10^{11}cells/ml. Less than 1% of these cells can form colonies on either minimal or rich medium. Cells could be stored at -20° for at least one month at this stage without appreciable loss in their in vitro activity.

For the measurement of in vitro DNA synthesis, reaction mixtures (0.05 ml) contained 0.5 μmole of dithiothreitol, 5 μmoles of KCl, 2.5 μmoles of morpholino propane sulfonic acid pH7.5, 0.1 μmole of ATP, 5 μmoles of $MgCl_2$, 5 nmoles of EDTA, 2 nmoles each of dATP, dGTP and dCTP, and 1 nmole of (α^{32}P)-dTTP (200-500 cpm per pmole). Between $5X10^6$ and 10^9 cells were placed in assay tubes at 0°, 50 μl of the reaction mixture was added, the cells were gently suspended and the tubes were placed in a 37° water bath for 15 min. The reactions were stopped by adding 0.5 ml of 0.5 N NaOH-1% sodium dodecyl sulfate and 0.2 ml of 0.1 M sodium pyrophosphate containing 1 mg per ml of calf thymus DNA. After 15 min at 80°, the tubes were filled with 4 ml of 10% trichloroacetic acid, and acid-insoluble material was collected on glass fiber filters and counted.

Results

E. coli D110 treated with 2 M sucrose incorporated radioactivity from base-labeled or α^{32}P -labeled dTTP into alkali-stable, DNase-sensitive material which banded in CsCl at the density of native E. coli DNA. The rate of incorporation of total nucleotide was 100-500 pmoles per 10^8 cells per 30 min at 37°. The in

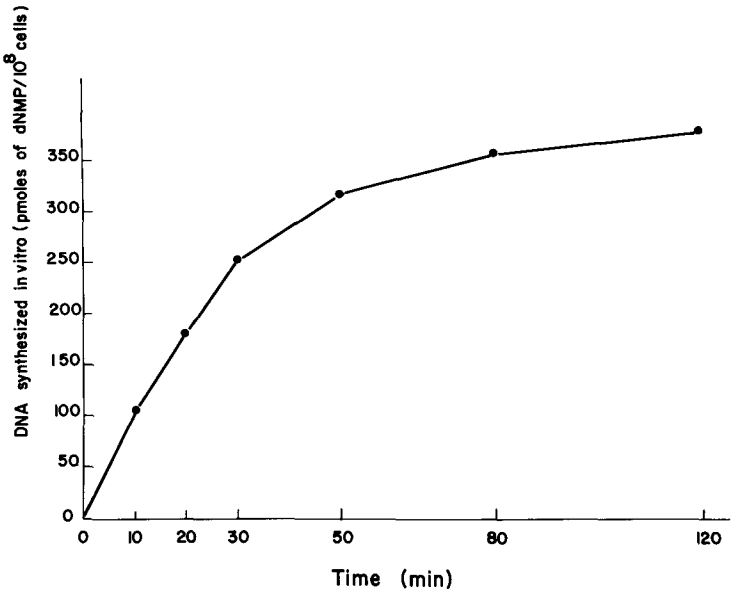


FIGURE 1: Kinetics of deoxynucleotide incorporation by plasmolysed cells at 37°

vivo rate of DNA replication of these cells is about 1500 pmoles of nucleotide per 10^8 cells per 30 min at 37°. Thus, the in vitro rate observed has varied between 6-30% of the in vivo rate.

The incorporation at 37° was linear for the first 20 min, gradually stopping at about 60 min when an amount of synthesis equivalent to 10-25% of the chromosome had occured (fig. 1). Synthesis at 30° continued for 120 min at a similar rate, stopping at 30-60% of the chromosome per cell. Addition of more deoxynucleoside triphosphates and ATP at 50 min, or inclusion throughout the incubation period of all four ribonucleoside triphosphates did not alter the kineti of the reaction. The incorporation was proportional to the number of cells between $5x10^6$ and 10^9 cells per assay. DNA labeled in vivo with ^3H -thymine is not degraded during a four hour incubation in vitro.

As shown in table 1, the activity of plasmolysed cells required Mg^{++}, all four deoxynucleotides and ATP. Deoxynucleoside monophosphates could substitute for the triphosphates, as observed with toluenized cells (9). The incorporation was inhibited by nalidixic acid, N-ethylmaleimide or high salt, but not by cyanide, azide, dinitrophenol, rifampicin or moderate concentrations of inorganic phosphate (table 1). The activity also was unaffected by the addition of 0.2 to 2% of the detergents Brij-58, Trition X-100 or Nonidet P40. Sodium deoxycholate (0.1%) inhibited the activity 70%.

TABLE 1

DNA SYNTHESIS IN PLASMOLYSED CELLS

Additions or Omissions	(α-^{32}P)dTMP Incorporation %	Additions or Omissions	(α-^{32}P)dTMP Incorporation %
Complete System	100	+ Nalidixic Acid (100 μg/ml)	21
-Mg^{2+}	14	+ DNase (10 μg)	8
-Mg^{2+} + EDTA (20 mM)	0	+ Trypsin (10 μg)	10
-rATP	8	+ Exonuclease III (120 units)	11
-3dNTP	13	+ NaCN (4 mM)	93
-dATP	22	+ Sodium Azide (4 mM)	92
-dCTP	25	+ Dinitrophenol (1 mM)	107
-dGTP	17	+ Potassium phosphate (20 mM)	98
-4dNTP-s + 4dNMP's (40 μM ea)	92	+ Potassium phosphate (40 mM)	30
-dTTP + dTMP	182	+ rifampicin (200 μg/ml)	100
-4dNTP's + dTMP	38	+ N-ethylmaleimide (4 mM)	6
		+ 4rNTP's	100

LEGEND

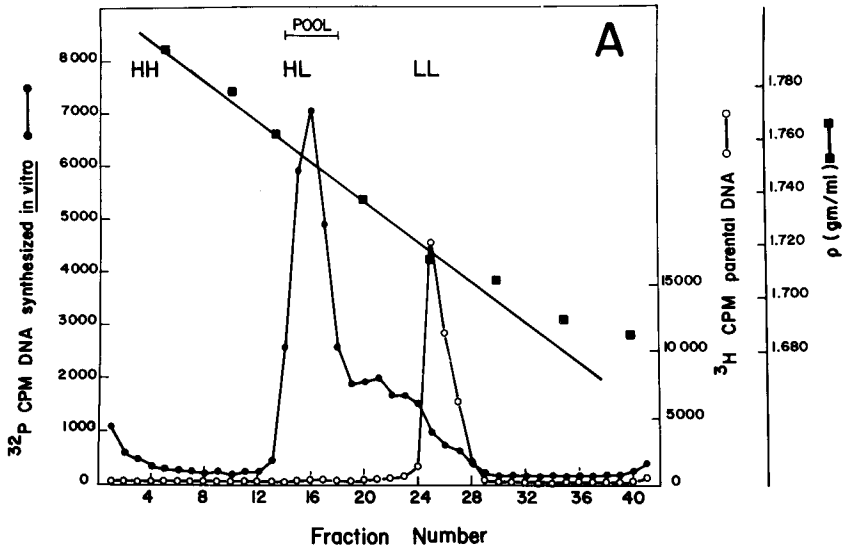
The complete system is the standard assay mixture described in Materials and Methods. 100% activity corresponded to 50 pmoles of dTMP (or 200 pmoles of total nucleotide) incorporated per 10^8 cells per 30 min.

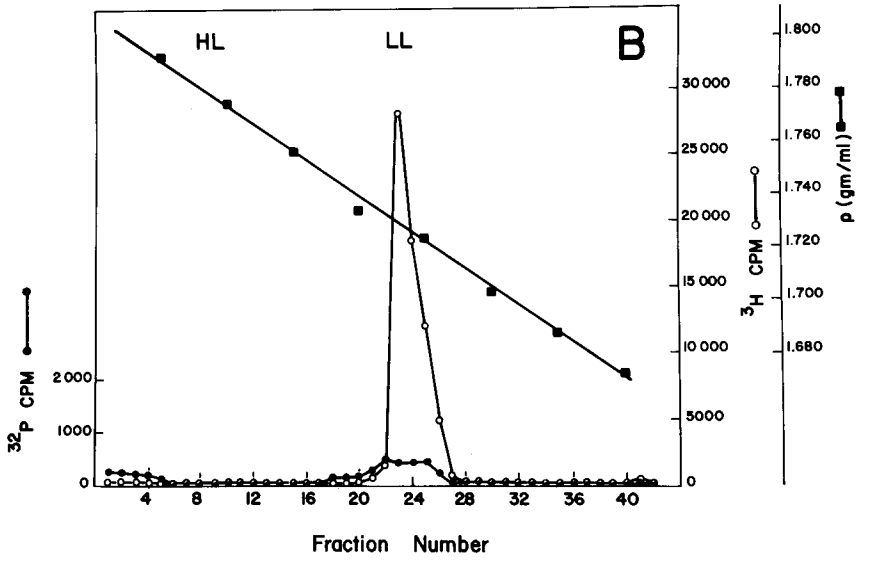
TABLE 2

IN VITRO DNA SYNTHESIS IN dna ts MUTANTS

E. coli strain used	Incorporation rate (pmoles total nucleotide/10^8 cells/30 min)		Rate at 43° / Rate at 25°
	25°	43°	
D110 (pol A$^-$, end$^-$)	331	897	2.71
1029 (dna B ts, polA$^-$, end$^-$, thy$^-$)	355	45	0.127
NY73 (dna G ts, polA$^-$)	280	167	0.60
BT1026 (dna E ts, polA$^-$, end$^-$, thy$^-$)	359	69	0.192

When temperature sensitive mutants, which immediately stop DNA replication on transfer to the non-permissive temperature *in vivo*, were used in the *in vitro* system, the incorporation also was temperature sensitive (table 2) suggesting that


A
POOL
HH
HL
LL
^{32}P CPM DNA synthesized in vitro
^{3}H CPM parental DNA
ρ (gm/ml)
Fraction Number


B
HL
LL
^{32}P CPM
^{3}H CPM
ρ (gm/ml)
Fraction Number

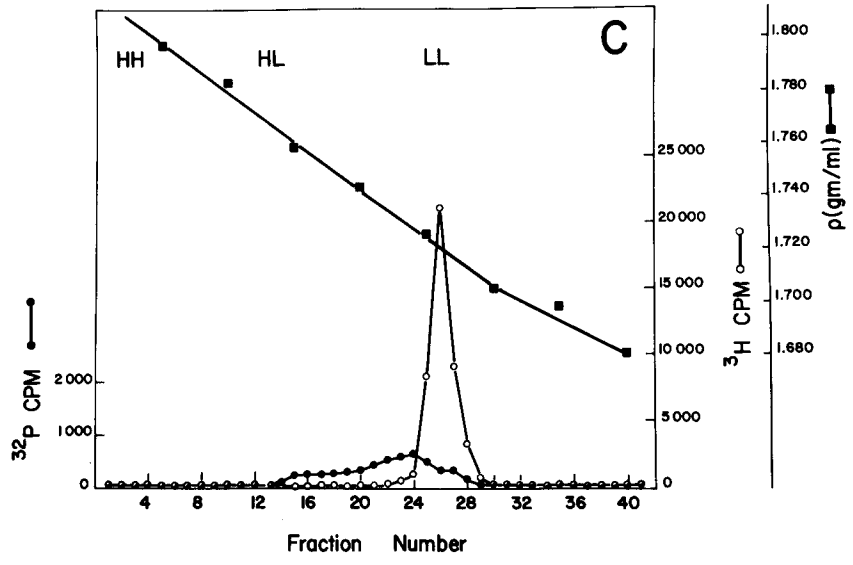

C
HH
HL
LL
^{32}P CPM
^{3}H CPM
ρ(gm/ml)
Fraction Number

FIGURE 2: Semiconservative DNA synthesis by plasmolysed cells. 3 x 10^9 plasmocells (E. coli D110) prelabeled with (^{3}H)-thymine were incubated for 20 min at 37° in 0.7 ml of the standard reaction mixture with (α^{32}P)dTTP replaced by dBrUTP and dATP replaced by (α^{32}P)-dATP. The cells incorporated 200 pmoles of total nucleotide per 10^8 cells after 20 min. The cells were lysed by the addition of 0.5 ml of 0.2 M EDTA, 0.2 ml of 1 M Tris chloride, pH 7.9, and 0.05 ml of 10% SDS and incubation at 37° for 10 min. The lysate was diluted to a total volume of 7 ml and the DNA sheared by gentle repeated passage through a pasteur pipette and sedimented to equilibrium in CsCl adjusted to an initial density of 1.740 gm/ml (32,000 rpm at 20° for 80 hours in 50 Ti rotor). Fractions (0.25 ml) were collected, precipitated with trichloroacetic acid, filtered and counted.

(A) Standard incubation conditions modified as described above.
(B) As in (A), but without ATP
(C) As in (A), but with 100 μg per ml of nalidixic acid.

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the in vitro DNA synthesis is due to replication rather than repair. This conclusion was confirmed by the finding that DNA synthesis was semiconservative.

DNA of plasmolysed cells labeled in vitro with dBrUTP and (α^{32}P)-dATP was sedimented to equilibrium in neutral CsCl gradients (fig. 2A). Seventy per cent of the ^{32}P was found in the half- heavy region where the product of semiconservative synthesis is expected to band. When fractions 14-18 (described in fig. 2A) were denatured and again sedimented to equilibrium in neutral CsCl gradients (fig. 3), most of the newly synthesized DNA was found at the density of fully BrU-labeled DNA, indicating that the half-heavy peak in fig. 2A was the

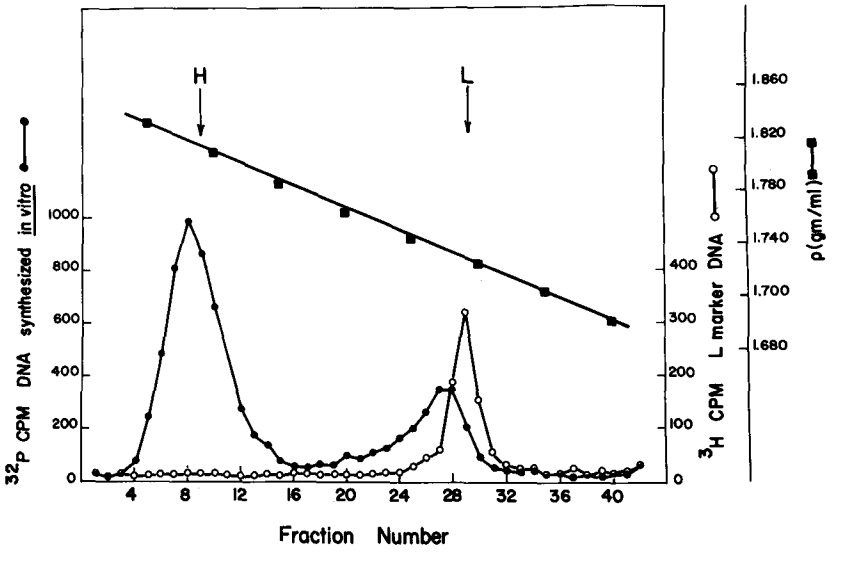

FIGURE 3: Density of denatured half-heavy DNA. Fractions 14-18 from Fig 1A were pooled, denatured in alkali, neutralized and sedimented to equilibrium in CsCl, adjusted to an initial density of 1.758 gm/ml (35,000 rpm at 20° for 72 hrs in 50 Ti rotor). Fractions (0.25 ml) were collected, acid-precipitated, filtered and counted.

result of semiconservative synthesis and was not due to extensive repair. This interpretation was confirmed further by the lack of formation of the half-heavy peak in the absence of ATP (fig. 2B) or in the presence of nalidixic acid (fig. 2C). The former is a specific requirement of replicative DNA synthesis (4, 9), while the latter is a specific inhibitor of DNA replication (11). The residual synthesis in each case was largely of the repair type.

Okazaki and co-workers (12) showed that the initial products of DNA replication *in vivo* sediment in alkali as small fragments of about 7-10S. Plasmolysed cells were pulse-labeled *in vitro* with (^{3}H)-dTTP for 20 sec. A 200-fold excess of cold dTTP was then added and the cells were allowed to continue synthesis for an additional 20 min period. The DNA from pulse-labeled as well as from pulse-

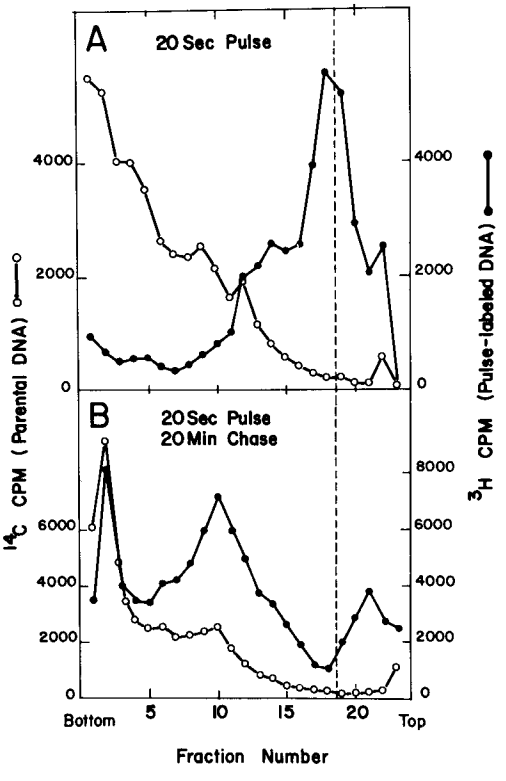

FIGURE 4: ^{14}C-thymine labeled plasmolysed cells (2 x 10^9) were incubated for 20 sec at 31° in 0.2 ml of the standard reaction mixture in which 2.2 nmoles of (^{3}H)-dTTP (4000 cpm/pmole) replaced (α^{32}P)-dTTP and 1.3 mM NAD was included. One reaction (A) was stopped at this point while to another (B) was added 550 nmoles of dTTP. After a further 20 min incubation (B) was also stopped. The reactions were halted by the addition of 20 µl of 1 M EDTA and 0.2 ml of 0.6 N NaOH containing 1.25 M NaCl. After 3 hrs at 37° the samples were poured gently onto a 5.0 ml 10 - 25% linear sucrose gradient containing 0.3 N NaOH, 0.7 M NaCl, and 1 mM EDTA over a 0.2 ml shelf of 60% sucrose. The gradients were centrifuged at 45,000 rpm at 4° for 105 min in the SW 50.1 rotor. Fractions of 0.23 ml were collected, acid precipitated, filtered and counted.

labeled and chased plasmolysed cells was extracted, denatured and sedimented in alkaline sucrose gradients (fig. 4). Pulse-labeled DNA was small (Fig. 4A) and could be chased into larger material, much of it as large as the parental DNA (fig. 4B). Thus, in this respect, the DNA synthesis carried out by plasmolysed cells resembles *in vivo* DNA replication.

If growing cells are treated with chloramphenicol, rounds of replication which are in progress are completed, but no new rounds are initiated (13). Such cells gradually stop replicating DNA. If these cells are then plasmolysed, they show markedly reduced activity *in vitro* when compared to cells which were in the log phase of growth at the time of harvest and plasmolysis (table 3). This suggests that the *in vitro* synthesis occurs primarily at replication forks and not at sites of repair. When the number of forks is reduced *in vivo* by chloramphenicol treatment, the *in vitro* rate of synthesis is reduced.

In order that plasmolysed cells be useful for studies of the enzymatic mechanism of DNA replication, it is desireable that they be freely permeable to macromolecules. Pancreatic DNase I (10 μg), exonuclease III (120 units), or trypsin (10 μg) included in the incubation mixture inhibited DNA synthesis by plasmolysed cells 92%, 89% and 90% respectively, while pancreatic DNase I degraded more than 95% of the parental DNA. This indicates that the site of DNA synthesis in plasmolysed cells is accessible to these proteins.

If cells are prepared as described in Materials and Methods, but with only 0.6M sucrose or no sucrose in the final buffer in which they are suspended, their

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EFFECT OF CHLORAMPHENICOL ON DNA SYNTHESIS

Chloramphenicol		DNA Synthesis	
vivo 0 μg/ml	*in vitro* 100 μg/ml	*in vivo* (cpm ^{14}C thymine/10^8 cells/5 min)	*in vitro* (pmoles/10^8 cells/30 min
-	-	827	200
-	+	827	156
+	-	90	12
+	+	90	11

GEND

E. coli strain D110 treated with chloramphenicol *in vivo* was incubated with aeration at 5 x 10^8 cells/ml in DC medium at 37° for 2 hrs. in the presence of 100 μg/ml of this drug. DNA synthesis *in vivo* was measured at the end of this period by the uptake of ^{14}C-thymine (30 μCi/mg) during a 5 min pulse. After centrifugation, cells were washed once with TG medium containing 100 μg/ml chloramphenicol and 2 μg/ml of thymine, then washed twice with 0.9% NaCl and plasmolysed as described under Material and Methods. Control cells were treated similarly except for the omission of chloramphenicol.

in vitro activity is less than 10% of that observed if 2M sucrose is used. Moreover only 5-10% of prelabeled DNA is degraded by added DNase I. Thus, the high sucrose treatment is necessary to make the cells permeable to both large and small molecules.

To determine what cellular components were lost to the medium on plasmolysis, cells were grown in DC medium with ^{14}C -thymine and either ^{3}H -uridine or ^{3}H -algal protein hydrolysate. The cells were harvested, washed and plasmolysed as described in Materials and Methods. The plasmolysed cell suspension was diluted into an incubation mixture, and after 15 min at 0°, centrifuged at low speed to pellet unbroken cells. While only 2% of acid-precipitable ^{14}C thymine was found in the supernatant, 14% of prelabeled protein and 15% of prelabeled RNA were released.

To determine whether the DNA was made within the plasmolysed cells or was synthesized on released DNA, such cells, after incubation in the standard reaction mixture, were sedimented through 10-30% sucrose gradients with a dense shelf of 5M CsCl and 60% sucrose (9 min at 17,000 rpm in the SW 50.1 rotor at 4°). Plasmolysed cells were found on the shelf while free DNA was recovered near the top of the gradient. Plasmolysed cells incorporated ($\alpha^{32}P$)-dTMP into particles sedimenting to the dense shelf.

Discussion

Plasmolysed cells utilize dNTP's or dNMP's to synthesize DNA in an ATP-dependent, nalidixic acid-sensitive, semiconservative process which requires the products of the dna B, dna E and dna G genes. The initial products of synthesis resemble Okazaki fragments and are eventually converted into larger DNA. We conclude that the synthesis closely resembles in vivo DNA replication.

The plasmolysed cells show considerable permeability to macromolecules. Since macromolecules apparently have access to the site of DNA replication, it may be possible to use this system to examine the effect of various proteins on the process.

Ben-Hamida and Gros (14) have shown that plasmolysed cells can carry out RNA and protein synthesis. We have confirmed this finding using cells prepared by our slightly different procedure. Thus, these cells may be useful in examining the relation between DNA, RNA and protein synthesis.

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