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INCORPORATION OF DEOXYRIBONUCLEOSIDE TRIPHOSPHATES INTO *ESCHERICHIA COLI* CELLS.[1]

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Abstract

Usually *Escherichia coli* cells are impermeable to deoxyribonucleoside triphosphates. In order to study the DNA synthesis, a mild treatment with toluene or with ether has been required to make the cell permeable to deoxyribonucleoside triphosphates. In this study some *E. coli* mutants were found to incorporate deoxyribonucleoside triphosphate directly without any treatment. *E. coli* cells grown at exponential stage could be just washed with tris-HCI buffer and directly used for deoxyribonucleoside triphosphate incorporation. The reaction requires Mg^{++}, all 4 of the deoxyribonucleoside triphosphates. The triphosphates cannot be replaced by monophosphates, ribonucleoside triphosphates or by deoxyribonucleosides. This condition was tested on strains H502, H560, ts_7, su 1 and C. Only strain C failed to show the incorporation and H560 required ATP. The incorporation of labeled H^3TTP into DNA in H502 cells is linear for 1 hr and optimum temperature is 39°C. Aeration stimulates the reaction. Alkaline sedimentation analysis of incorporation product showed that the deoxyribonucleoside triphosphates were incorporated into DNA.

INTRODUCTION

It has been generally believed that *E. coli* cells are impermeable to deoxyribonucleoside triphosphates (*d*NTPs) (Buttin and Kornberg 1966). Therefore, investigations of deoxyribonucleic acid synthesis have only been limited in exploring the properties of DNA polymerase purified from cell-free extracts or the behavior *in vivo* systems which are impermeable to the *d*NTPs, the substrates for enzymatic polymerization. In order to acquire additional insights into the mechanism of DNA synthesis and the factors that influence it, it is important to make cells, with functioning replicating machinery, permeable to *d*NTPs.

Several attempts have been made in the past years to detect incorporation of *d*NTPs either by disrupted cells or by cells whose permeability was altered

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in some way (Smith *et al.*, 1970; Buttin and Kornberg, 1966; Denhardt, 1968; and Knipers and Strätling, 1970). Buttin and Kornberg (1966), for instance, observed some incorporation in EDTA-treated wild type cells. This incorporation, however, disappeared with the use of mutants devoid of endonuclease activity. Moses and Richardson (1970) and Mordoh *et al.* (1970) indicated that in contrast to EDTA, a mild treatment with toluene makes the cell permeable to *d*NTPs without destroying the capability for DNA synthesis. Vosberg and Hoffman-Berling (1971) have also made cells permeable to nucleotides by brief treatment with ether and have used these cells for the study of DNA synthesis.

In order to study the DNA synthesis in toluenized cells, the incorporation of *d*NTPs to toluenized cells was examined in our laboratory. When untreated cells were used for control, it was found that many *E. coli* mutants were permeable to *d*NTPs directly without any treatment and the rate of *d*NTP incorporation in untreated cells were comparable to that of treated cells. It would be interesting to see if these *d*NTPs are really incorporated into DNA. In this investigation the conditions for incorporation in intact *E. coli* cells were studied and the product of this incorporation was also characterized.

Materials and Methods

Bacteria: The following 5 strains of *Escherichia coli* were used in these experiments. (1) H502 ($uvrA^-$, $thyA^-$, end 1^-) is a N-methyl-N-Nitro-N-nitrosoguanidine (NNG) mutant of HF 4704 (Linquist and Sinsheimer, 1967) (2) H560 (F^+, polA, $strA^-$, end 1^-, tsc^-) is a sexual recombinant from a cross of a tsx^- derivative of strain p3478 (De Lucia and Cairns, 1969). (3) C is usual host for ϕX174, BTCC No. 122. (4) Ts_7 is a temperature-sensitive radiation-sensitive mutant, which was derived from TAU-bar (Pauling and Hamm, 1968). (5) Su 1 is a suppressor derivative and is believed to insert serine at the site of the nonsense cordon (Funk and Sinsheimer, 1970). All bacteria strains were kindly supplied by R. L. Sinsheimer.

Media: For the growth of H502, H560, C and ts_7, TPA medium was used. TPA medium is TPG (Sinsheimer *et al.*, 1962) plus 5 g per liter of an amino acid mixture (special amino acid mixture, Nutritional Biochemicals Corp., Cleveland, Ohio). For the growth of su 1 TPA supplemented with 30 mg cytidine HCl, 50 mg proline, 50 mg methionine, 50 mg tryptophane, and 40 mg thymidine per liter was used.

Chemicals: Thymidine-5′-triphosphate-(methyl-H^3) was purchased from Schwarz/Mann, Dickinson and Co., its specific activity was 16.8 c/m mole. Deoxyadenosine-5'-triphosphate-α-p^{32} was purchased from International Chemical and Nuclear Corp., its specific activity was 3.6 c/m mole.

Tris, tris-EDTA and toluene treatment of cells: Overnight cultures of cells grown on growth media were regrown with vigorous shaking at 37°C on the same medium from 8×10^7 cells/ml to 4×10^8 cells/ml. The cells were harvested by centrifugation, washed twice with cold 0.01 M tris-HCl buffer pH 8.0, and finally concentrated 10 fold in the same buffer. The preparation was called tris-treated cells. Toluene-treated cells were made by adding 1% of toluene into the tris-treated cells, and the mixture was maintained in ice for 15 min with intermittent agitation. After the treatment, the cells were washed with tris-buffer and finally suspended in the same buffer. Tris-EDTA cells were made by adding 0.2 mM EDTA to tris-treated cells and the suspension was incubated at 37°C for 10 min. and then washed with tris-buffer. These suspensions were directly used for assay.

Incorporation of dNTP: The method used was described by Smith *et al.* (1970) with some modifications. 0.2 ml of reaction mixture contained 0.05 M tris-HCl buffer pH 8.0, 0.005 M $MgCl_2$, 0.09 M KCl, 0.01 M β-mercaptoethanol, 3.5×10^{-5}M *d*GTP, *d*ATP, *d*CTP, 0.001 M ATP, and 0.1 μc/ml of H^3-TTP. The reaction was initiated by addition of 0.1 ml cell preparations (4×10^8 cells/ml) and terminated after 30 min by addition of 0.2 ml of 1 M NaOH—0.02 M EDTA. The mixture was heated for 30 seconds at 100°C cooled to 0°C before 0.2 ml of 1 M HCl and 0.5 ml of 10 per cent ice-cold TCA were added, the sample filtered on Whatman GF/A glass filters, rinsed with 10 ml of ice-cold 0.5 per cent TCA and then 10 ml ice-cold ethanol, dried and placed in a vial containing 5 ml toluene-POPOP-PPO scintillation fluid. Radioactivity was measured with a Nuclear-Chicago liquid scintillation counter.

Alkaline sedimentation analysis of DNA: Cells (30 ml) were grown in H^3-thymine for two and half generations, harvested, concentrated and washed with 0.01 M tris-HCl buffer pH 8.0. Cells were added to a reaction mixture described as above, except cold *d*ATP and H^3-TTP were replaced with cold TTP and α-p^{32}-*d*ATP respectively. Total volume of reaction mixture was 1 ml. It was incubated at 36°C for 40 min. The reaction was terminated by addition of 1 ml of 0.5 M sodium azide (NaN_3) and freezed in dry-ice-methanol. After thawing, the cells were washed twice with cold tris-EDTA-NaN_3 (0.01 M tris-HCl pH 8.0, 0.005 M EDTA, 0.5 M NaN_3) and finally suspended in 0.6 ml of the same solution, added 60 μl of 0.4 M EDTA. 120 μl of 500 μg/ml *t*RNA, 120 μl of 2 mg/ml lysozyme and then incubated at 36°C for 20 min to digest the cell wall. The lysate was frozen and thawed twice then added 400 μl salkosyl, gently shaked till the lysate became clear. Then RNA in the lysate was digested by adding 120 μl 1 mg/ml RNase and incubating at 36°C for 30 min. Finally the protein in lysate was digested by adding 120 μl 1 mg/ml pronase and incubating at 36°C for 4 hrs. An aliquot of the lysate was placed on a

linear 0.7 M NaCl–0.3 M NaOH–1 mM EDTA 30–70% sucrose gradient, and centrifuged for 3.5 hrs at 35,000 rpm in Spinco type SW 50.1 rotor at 4°C. Three drops fraction were collected on Whatman 3 MM filters (2.4 cm) from the bottom of the tube and the filters were washed with 5% TCA, then with methanol. After drying the radioactivity was counted in a toluene scintillation fluid.

Results

Incorporation of dNTPs with different strains of E. coli cells:

The incorporation of *d*NTP was compared in tris buffer, tris-EDTA and toluene treated cells of *E. coli.* It was found that some *E. coli* strains could directly incorporate the label from α-p^{32}-*d*ATP or H^3-TTP into a TCA insoluble fraction without any treatment. However the rate of incorporation was different among different strains of *E. coli* and different treatments. As indicated in Table 1, after EDTA treatment, all five strains of *E. coli* cells completely lost the ability to incorporate *d*NTPs. When cells were treated with toluene and tris, four strains showed the incorporation. Strain H560 and H502 were the best and strains ts_7 and su 1 came next whereas C was almost impermeable. When incorporation was compared between toluene-treated cells with different strains, the behavior was also different. With strains ts_7 or su 1 the incorporation in tris-treated cells was better than that in toluene-treated cells. However, with H560 the situation was reversed. There was no difference between toluene-treated and tris treated cells when strain H502 was examined.

Table 1. *Comparison of dNTP incorporation in tris-, tris-EDTA and toluene treated cells of various strains of E. coli. Cells were grown in 20 ml of growth medium to a concentration of 4×10^8 cells/ml, washed with 0.01 M tris buffer pH 8.0, concentrated 10 fold in same buffer, then separated to three parts, they were treated with toluene, tris or tris-EDTA respectively. 0.1 ml of cell suspension was taken and mixed with reaction mixture. The reaction was conducted at 36°C for 30 min. *The activity was expressed as cpm of H^3-TTP incorporated into 4×10^8 cells.*

Strains	toluene-treated cells	tris-treated cells	tris-EDTA treated cells
C	41	28	25
su 1	259	779	17
ts_7	905	1296	48
H502	1585	1635	26
H560	1876	985	15

Characteristics of the reaction:

Incorporation of *d*NTP in normal H502 cells has been followed by incorporation of H^3-TTP into acid-precipitable materials. Among the components of the standard incubation mixture, Mg^{++} was absolutely required. In its absence, no incorporation was observed. The reaction required the presence of all four *d*NTPs. In the absence of *d*NTPs but with deoxyribonucleoside monophosphates present, no incorporation of H^3-TTP occurred, indicating that a deoxyribonucleoside monophosphates did not replace the corresponding *d*NTP under this condition. Ribonucleoside triphosphates or deoxyribonucleosides also failed to substitute for *d*NTPs (Table 2). The rate of nucleotide incorporation was the same whether the labeled triphosphates was α-p^{32}-*d*ATP or H^3-TTP. ATP was not required for H502 cells. Ts_7 and su 1 were identical to H502 with respect to the requirements of *d*NTPs incorporation. In the case of H560 cells, ATP was additionally required. Buffer used in the reaction mixture was also very important. When 0.01 M phosphate buffer pH 7.5 was replaced with tris-HCl buffer in the above reaction, no incorporation was observed for H560 cells but no difference was detected for H502 cells.

Table 2. *Characteristics of dNTP incorporation in E. coli strain H502. Incorporation of H^3-TTP into acid-insoluble fraction was measured.*

System	Activtty (%)	System	Activity (%)
Complete	100	−*d*CTP	9
−ATP	100	−4*d*NTP	2
−Mg++	4	−4*d*NTP, +4*d*NMP	0
−*d*GTP	11	−4*d*NTP, +4NTP	2
−TTP	6	−4*d*NTP, +4*d*N	0

The efficiency of *d*NTP incorporation was temperature-and time-dependent. The incorporation of labelled H^3-TTP into DNA in H502 cells was linear for 1 hr (Fig. 1). No incorporation was observed when the reaction was conducted below 15°C. Incorporation after a 30-min incubation was maximal at 39°C, however, there was not much difference at temperature range 33°–42°C (Table 3).

The extent of the reaction is directly proportional to the concentration of the cell suspensions from 6×10^7 cells/ml to 9×10^8 cell/ml per reaction mixture (Fig. 2). The aeration of reaction mixture was also conducted by air bubbling. As indicated in Table 3 bubbling stimulated the *d*NTP incorporation.

Characterization of the DNA product:

E. coli was grown for several generations in the presence of H^3-thymidine. The cells were then washed with 0.01 M tris-HCl pH 8.0 and incubated in the

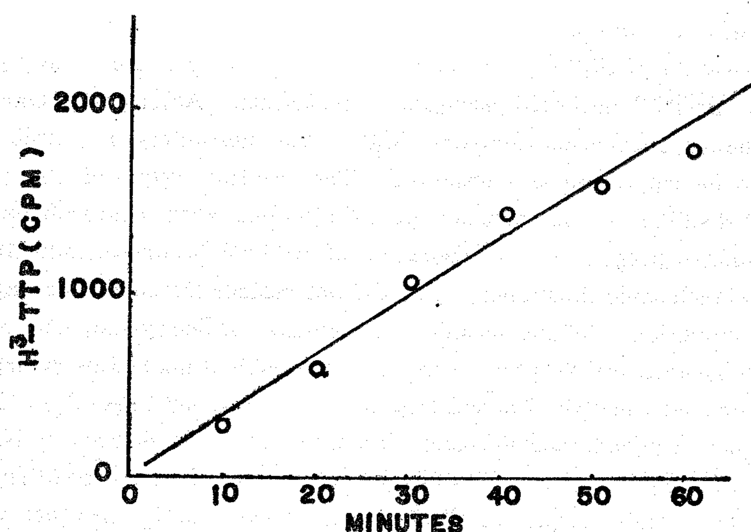

Fig. 1. Rate of *d*NTP incorporation in H502 cells. Cells were washed twice in 0.01 M Tris-HCl and the assay was processed as indicated in "Method".

Table 3. *Effect of temperature and aeratian on dNTP incorporation in E. coli H502. Aeration was conducted by bubbling air through reaction mixtures.*

Temperature (C)	15°	20°	30°	33°	36°	39°	42°
H^3-TTP incorporated (cpm)	0	45	1,250	2,153	2,477	3,156	2,234

aeration	bubbling	without bubbling
H^3-TTP incorporated (cpm)	1,630	998

standard reaction mixture containing α-*p*-^{32}-*d*ATP. DNA was extracted and analyzed with alkaline sucrose gradient centrifugation. As shown in Fig. 3. the newly synthesized DNA was distributed over the same range as the prelabelled DNA.

DISCUSSION

Buttin and Kornberg (1966) have found two ways in which *d*NTP permeability of *E. coli* can be altered so that the intracellular synthesis of DNA can be studied. One of them involved the exposure of cells to tris-buffer in the presence of EDTA (tris-EDTA) and the other in the presence of Mn^{++} (tris-Mn^{++}). All four of the deoxyribonucleoside triphosphates are required, and cannot be replaced by their monophosphates or by their correspondent ribonucleoside triphosphates. They concluded that the utilization of *d*NTPs after tris-EDTA treatment is the result of (a) increased permeability and (b) the exposure of priming points for polymerase action on the cellular DNA. The specific effect on Mn^{++} in the tris-Mn^{++} treatment is not explained.

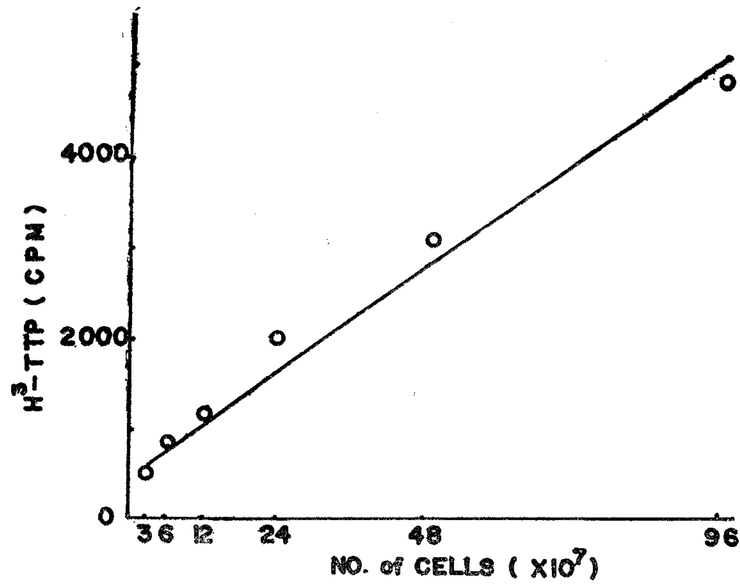

Fig. 2. Effect of cell concentration of *d*NTP incorporation in H502 cells. Cells were washed with 0.01 M tris-HCl buffer and concentrated to 9×10^8 cells per ml. From this stock cells were dilute to indicated concentrations and the assay was processed as indicated in "Method".

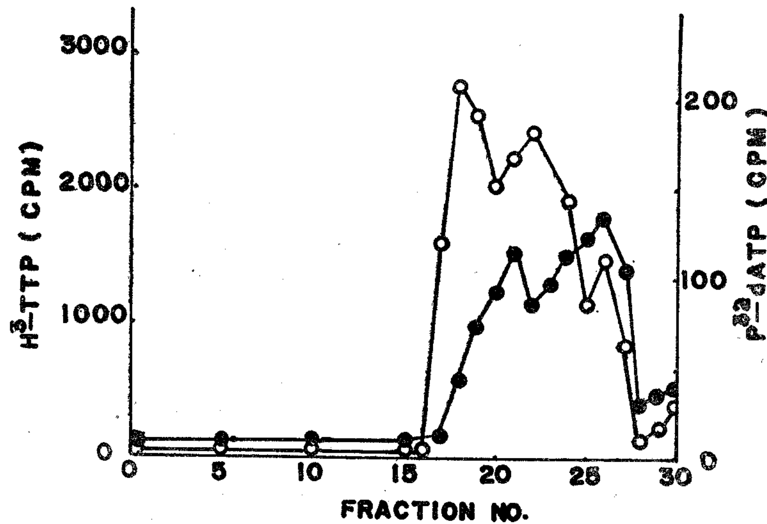

Fig. 3. Alkaline sedimentation analysis of the product of *d*NTP incorporation in H502 cells. The incorporation reaction carried out for 40 min with 1 ml sample of tris-HCl treated cells was stopped by adding 1 ml of 0.5 M NaN_3. The DNA was extracted and a 1.0 ml sample was layered on top of an alkaline sucrose gradient and processed as indicated in "Methods". o——o——o = H^3-TTP, •——•——• = p^{32}-*d*ATP.

Vosberg and Hoffmann-Berling (1971) used treatment with ether and Moses and Richardson (1970) used toluene to render *E. coli* cells permeable to low

molecular weight substances. After these treatments the cells remain intact in appearence but are incapable of forming colonies. When incubated with ATP, four *d*NTPs, and Mg^{++} they synthesize DNA.

The present study provided evidence that the cells of some *E. coli* mutants are permeable to *d*NTPs directly without any treatments, and also permits the utilization of *d*NTPs for intracellular DNA synthesis. The reaction required Mg^{++} and four *d*NTPs. This kind of requirement is also different from that of tris-EDTA and toluene systems. In tris-EDTA system Mg^{++} is not required and in toluene-system additional ATP is required.

The rate of incorporation of different strains of *E. coli* was compared in three systems. It was found that tris-EDTA treatment failed to make all five strains of cells permeable to *d*NTP. Furthermore it destroyed the permeability of cells which were permeable in other condition. Tris-buffer treatment improved *d*NTP incorporations for all bacterial strains except C. Toluene treatment inhanced the incorporation for H560 further but not the other strains tested. It is also interesting that when H560 and H502 were treated with tris-buffer system, the ATP was required for H560, but not for H502. When tris-buffer was replaced with phosphate buffer H560 failed to show any incorporation, but there was no effect to H502. Evidently there were many distinctive differences of membrane structure among various strains of *E. coli*. It would seem that if more were known about the chemistry of membrane structure, agents such as tris and EDTA could be used more effectively to which the cell membrane is ordinarily impermeable.

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Deoxyribonucleoside triphosphates 滲入大腸菌細胞之研究

郭　宗　德

一般大腸菌細胞不能使 deoxyribonucleoside triphosphates (*d*NTP) 滲入。因此在研究 DNA 合成之過程中常用 toluene 或 ether 處理使其變爲能滲透 *d*NTP 之細胞。本研究發現有些大腸菌變異株不經任何處理就能使 *d*NTP 直接滲透進入細菌細胞。細菌用 0.01 M tris-HCl pH 8.0 之緩衝液清洗後可直接用來做實驗。*d*NTP 滲入細胞之速度依細菌品係之不同而異。用五品係之大腸菌比較其滲透情形發現以 H560 及 H502 最好，其次爲 ts_7 和 su 1 而 C 變異株則完全不能使 *d*NTP 滲入。整個反應需 Mg^{++} 離子，四種 deoxyribonucleoside triposphates。Monophosphates, ribonucleoside triphosphates 或者 deoxyribonucleosides 均不能代替 *d*NTP。在反應中 H502, ts_7 和 su 1 不要求 ATP，但 H560 則需要 ATP。*d*NTP 滲入細胞之速度在一小時內成直線關係，又與細菌濃度成正比。反應最適溫度是39°C。通氣可加速反應。滲入細胞內之 *d*NTP 利用 alkaline sedimentation 分析結果證明 *d*NTP 確能加入細菌 DNA 中。