Incorporation of nucleotide tracers into nucleic acids in permeabilized cells and cellular homogenates

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The validity of permeabilized cells as a model of DNA and RNA synthesis was studied with the use of mouse S-49 lymphoblastoma cells rendered permeable by exposure to L- α -lysophosphatidylcholine. The permeabilized cells readily incorporated exogenously supplied cytosine and uracil nucleotides into HClO₄-insoluble macromolecular material. However, the incorporation of these tracers did not require the three other complementary nucleotides, and adenine, guanine or thymine nucleotide tracers were incorporated at much lower rates. These results, which were also obtained with permeabilized Abelsohn-leukaemia-virus-transformed mouse macrophages, mouse neuroblastoma cells and S-49 lymphoblastoma homogenates, are inconsistent with semi-conservative DNA replication or RNA transcription; rather, they suggest the involvement of terminal nucleotidyltransferase(s) that mediate the incorporation of uracil and cytosine nucleotides. DNA synthesis was restored when permeabilized cells or cellular homogenates were supplemented with denatured salmon testes DNA. These results suggest that endogenous cellular DNA is impaired in its function as a template for DNA replication and transcription *in vitro*. Metabolic channelling or compartmentation of nucleic-acid-precursor pathways could not be demonstrated in the permeabilized cells.

INTRODUCTION

The relationship between the biosynthesis, size and compartmentation of cellular nucleotide pools and their utilization in eukaryotic DNA replication has been studied with intact cells (Taheri *et al.*, 1981; Nicander & Reichard, 1983), permeabilized cells (Reddy & Pardee, 1982) and isolated enzyme complexes (Wickremasinghe *et al.*, 1982; Noguchi *et al.*, 1983). Distinct separate nucleotide pools might exist in different cellular structures, e.g. nuclear or mitochondrial pools (Bestwick & Mathews, 1982), or, concomitantly, such pools might be created at the molecular level in multi-step biosynthetic processes that serve different cellular functions. It was proposed (Taheri *et al.*, 1981; Reddy & Pardee, 1982) that a multienzyme complex channels distal precursors and maintains high concentrations of the ultimate substrates for DNA synthesis, deoxyribonucleoside triphosphates, at the replication fork.

Kinetic study of nucleotide-precursor pools for DNA and RNA synthesis in intact mouse lymphoblastoma S-49 cells demonstrated the existence of very small functional guanine-nucleotide pools that provided the immediate precursors for nucleic acid synthesis, as distinct from the general cellular pool (Nguyen & Sadée, 1986). We decided here to test the possible role of metabolic channelling and compartmentation of nucleic-acid-precursor pools with the use of permeabilized cells. This system was used by Reddy & Pardee (1982) to demonstrate that the reduction of cytidine diphosphate is the first enzymic reaction that channels cytosine nucleotides into DNA. However, the validity of this conclusion was questioned by Spyrou & Reichard (1983),

who proved that ribonucleoside diphosphates were incorporated into RNA instead. Furthermore, our own initial results revealed that the incorporation of certain tracers into acid-insoluble macromolecules was not dependent upon the presence of all four complementary bases as should be expected for DNA and RNA synthesis. We therefore studied in detail the fate of nucleic-acid-precursor tracers in permeabilized cell systems.

EXPERIMENTAL

Chemicals and radiochemicals

The following chemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.: lyso-PtdCho (type I), NaN₃, 2-mercaptoethanol, DTT, salmon testes DNA, PEP, PEP kinase, Hepes, ribo- and deoxyribo-nucleoside 5'-phosphates (AMP, CMP, GMP, UMP; ADP, CDP, GDP, UDP; ATP, CTP, GTP, UTP; dATP, dCTP, dGTP and dTTP), ribo- and deoxyribo-nucleosides (rA, rC, rG, rU, dA, dC, dG, dT), DNAase I (EC 3.3.4.5) type I, RNAase A type I-AS, RNAase T₂ (EC 3.1.27.1) grade VI, venom phosphodiesterase (EC 3.1.4.1) type VI, nuclease P₁ from *Penicillium citrinum*, alkaline phosphatase (EC 3.1.3.1) type III and BSA.

Amersham Corp. (Arlington Heights, IL, U.S.A.) supplied: [2-³H]AMP (15.8 Ci/mmol), [5-³H]CMP (13 Ci/mmol), [5-³H]UMP (11.2 Ci/mmol), [5-³H]CDP (16 Ci/mmol), [8-³H]GDP (10.6 Ci/mmol), [2,8-³H]ATP (31.7 Ci/mmol), [5-³H]dCTP (19 Ci/mmol) and [methyl-³H]dTTP (45 Ci/mmol). [8-³H]GTP (13 Ci/mmol), [5-³H]UTP (30 Ci/mmol) and [8-³H]dGTP (5 Ci/mmol)

Abbreviations used: lyso-PtdCho, L- α -lysophosphatidylcholine; DTT, dithiothreitol; PEP, phosphoenolpyruvate; DNAase, deoxyribonuclease; RNAase, ribonuclease; BSA, bovine serum albumin; DME, Dulbecco's modified Eagle medium; HAT, hypoxanthine/adenine/thymidine.

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were purchased from ICN Radiochemicals, Irvine, CA, U.S.A., and [8,5'- ^3H]GMP (38 Ci/mmol) and [methyl- ^3H]thymidine (50 Ci/mmol) were from New England Nuclear Corp., Boston, MA, U.S.A.

Cell-culture media and their supplements were obtained from the Cell Culture Facility at this University.

Cell cultures

Mouse lymphoblastoma S-49 cells were harvested during their exponential growth $[(1-1.7) \times 10^6 \text{ cells/ml}]$ in DME supplemented with 10% (v/v) horse serum and glucose (4.5 g/l). Abelson-leukaemia-virus-transformed mouse macrophages RAW 264.7 (Rashke *et al.*, 1978) were grown in monolayer in DME medium supplemented with 10% (v/v) foetal-calf serum and were collected at confluency $[(6-7) \times 10^5 \text{ cells/cm}^2]$. Mouse neuroblastoma $\times$ rat glioma NG-108 hybrid cells (Klee & Nirenberg, 1974) were grown in DME/HAT medium enriched with 10% fetal bovine serum and were harvested at confluency density of $1.5 \times 10^5 \text{ cells/cm}^2$. All cell cultures were seeded 2-4 days before harvest and maintained in an atmosphere of CO_2/air (1:9) and 95% humidity.

Cell permeabilization

S-49, RAW 264.7 or NG-108 cells were permeabilized as described by Miller *et al.* (1978), with the following modifications. The cells were collected by centrifuga-

tion at 320 g for 5 min at 4 °C, washed twice with 30 ml of ice-cold 150 mM-sucrose/80 mM-KCl/35 mM-Hepes/5 mM-potassium phosphate/5 mM-MgCl₂ buffer, pH 7.4, and subsequently suspended in the same buffer at a density of 5×10^7 – $5 \times 10^8 \text{ cells/ml}$. lysoPtdCho solution (2 mg/ml) was added to yield a final concentration of 150 $\mu\text{g/ml}$, and the mixture was incubated for 2 min at 4 °C. Under those conditions, 95–100% of the cells were rendered permeable, as judged by Trypan Blue exclusion. Alternatively, S-49 cells were permeabilized by a 15 min cold-shock with 10 mM-Tris/HCl/63 mM-sucrose/1 mM-EDTA/30 mM-2-mercaptoethanol/4 mM-MgCl₂ buffer, pH 7.8 (Berger & Johnson, 1976). Complete cell homogenates were obtained by subjecting the ice-cold cell suspensions to three 20 s bursts with a Brinkman blender.

Incorporation of precursors into nucleic acids

Reactions were carried out in 1.5 ml Eppendorf plastic tubes with $(1-5) \times 10^6$ permeabilized cells or the equivalent cell homogenate in 10–25 μl of the permeabilizing buffer diluted to 100 μl with 50 mM-Hepes/7.5 mM potassium phosphate/10 mM-MgCl₂ buffer, pH 7.4. The final reaction mixture contained 5 mM-DTT, 3 mM-ATP, appropriate nucleotides that were added each to a final concentration of 0.2 mM, and 10–20 μCi of the radioactive nucleotide tracer/ml. The mixture

Fig. 1. Rates of $[^3\text{H}]$ dCTP (○) or $[^3\text{H}]$ CDP (●) incorporation into macromolecules of permeabilized S-49 cells

(a) Permeabilized cells ($1.5 \times 10^6/100 \mu\text{l}$) were incubated for 60 min with 3 mM-ATP, 0.33 mM-CTP, 0.33 mM-GTP, 10 mM-PEP, 8 mM-DTT and increasing concentrations of each of the four complementary dNTPs with $[^3\text{H}]$ dCTP (○) or NDPs with $[^3\text{H}]$ CDP (●) radioactive tracer at 20 $\mu\text{l/ml}$. The HClO_4 -insoluble radioactivity was determined in duplicate samples, and incorporation rate was calculated from specific radioactivity of the tracer nucleotide. (b) The double-reciprocal plots indicate (○) a V_{max} of 2.2 pmol/min per 10^6 cells and a K_m of 0.06 mM for dCTP incorporation (with a linear correlation coefficient, r^2 , of 0.99), and (●) a V_{max} of 2.9 pmol/min per 10^6 cells and a K_m of 0.10 mM for CDP incorporation (with $r^2 = 0.96$).

containing all four deoxyribonucleoside triphosphates, ribonucleoside triphosphates, ribonucleoside diphosphates and ribonucleoside monophosphates were termed dNTP, NTP, NDP and NMP reaction complement respectively. The NDP reaction mixture tested for DNA synthesis contained dTDP in place of UDP to bypass the thymidylate synthetase pathway (Reddy & Pardee, 1982). Incorporation of nucleoside triphosphates into nucleic acids was carried out in the presence of 10 mM-PEP (Miller *et al.*, 1978; Reddy & Pardee, 1982). Salmon testes DNA at 1 mg/ml in 10 mM-Tris/5 mM-MgCl₂ buffer, pH 7.4, was denatured at 100 °C for 10 min, quenched in ice/water and included in the incubation mixture at 100 µg/ml as an extracellular DNA template. The components were combined at 4 °C, and the reactions were started by transferring the tubes to a 37 °C water bath. After appropriate incubation times, a solution of a carrier DNA and RNA (100 µg/100 µl) was added and cellular macromolecules were precipitated with 40 µl of ice-cold 60% (w/v) HClO₄ supplemented with 1% Na₄P₂O₇. The mixtures were then left for 30 min at 4 °C, centrifuged for 15 s at 12800 *g* in a Beckman Microfuge, and the pellets were washed twice with 750 µl of ice-cold 5% HClO₄ solution supplemented with 1% Na₄P₂O₇. The precipitates were subsequently incubated overnight at room temperature with 200 µl of 0.3 M-NaOH. Then, after chilling the samples, 40 µl of ice-cold 60% HClO₄/1% Na₄P₂O₇ was added to each digest, and the mixtures were left on ice for the ensuing 30 min. After centrifugation, alkali-hydrolysable nucleic acids were recovered in the supernatants, the pellets were dissolved in 300 µl of 0.3 M-NaOH at 50 °C, and the radioactivity of both fractions was counted in 7 ml of Aquasol with external standardization to obtain d.p.m.

A series of experiments was performed to compare tracer incorporation into DNA after prelabelling with [³H]dT before permeabilization and after direct labelling with [³H]dTTP in permeabilized cells. S-49 cells were incubated in cold (4 °C) fresh DME medium supplemented with 10% horse serum containing [³H]dT at 10 µCi/ml and 4 °C for 30 min, washed, and permeabilized with lysoPtdCho as described above. Prelabelled intact or permeabilized cells (5 × 10⁶) in 50 µl of the permeabilizing buffer were diluted to 100 µl with 50 mM-Hepes/7.5 mM-potassium phosphate/10 mM-MgCl₂ buffer, pH 7.4, containing 2 mM-DTT, 20 mM-PEP and 6 mM-ATP. The buffer used for dilution of unlabelled cells also contained [³H]dT at 10 µCi/µl or [³H]dTTP at 12 µCi/ml for intact or permeabilized cells respectively. The components were combined at 0 °C, then incubated at 37 °C, and DNA incorporation of tracer was determined as described above. Acid-soluble fractions of each sample were also assayed by h.p.l.c. for tracer distribution between thymine-nucleotide pools as described previously (Lee *et al.*, 1985).

Enzymic hydrolysis of acid-precipitable nucleic acids

The total acid-precipitable material was recovered as described above, dissolved in 100 µl of 50 mM-Hepes/7.5 mM-potassium phosphate/10 mM-MgCl₂/0.01% NaN₃ buffer, pH 7.4, and incubated overnight at 37 °C with 20 Kunitz units of DNAase I, 1 unit of RNAase A and 0.2 unit of RNAase T₂. Then the samples were adjusted to pH 8.5 with 1 M-NaOH and the mixture was further digested for 2 h at 37 °C with 0.005 unit of snake-venom phosphodiesterase, 0.01 unit of nuclease P₁

Table 1. Requirements for dCTP or CDP incorporation into macromolecules of lysoPtdCho-permeabilized S-49 cells

The permeabilized cells (4 × 10⁶/100 µl sample) were incubated (30 min, 37 °C) in the complete mixture containing 3 mM-ATP, 0.033 mM-CTP, 0.033 mM-GTP, 10 mM-PEP, 8 mM-DTT or, alternatively, dNTP reaction complement (dATP, dCTP, dGTP, dTTP at 0.2 mM, each with 1 µCi of [³H]dCTP tracer) or NDP reaction complement (ADP, CDP, GDP, dTDP at 0.2 mM, each with 1 µCi of [³H]CDP tracer). Some factors or substrates were omitted from the complete mixture, as indicated. The HClO₄-insoluble radioactivity was determined in duplicate samples and expressed as a percentage (mean ± range) of [³H]dCTP or [³H]CDP incorporation in the complete mixture (1.56 ± 0.17 or 1.51 ± 0.01 pmol/min per 10⁶ cells respectively). The data are from a single experiment; independent experiments yielded similar results with a [³H]dCTP incorporation rate of 2.5 ± 0.9 pmol/min per 10⁶ cells (mean ± s.d.; 12 expts.) and a [³H]CDP incorporation rate of 2.4 ± 0.9 pmol/min per 10⁶ cells (mean ± s.d.; eight expts.).

Composition of the reaction mixture	Incorporation (%)
[³ H]dCTP tracer in	
complete dNTP complement	100
– CTP, – GTP, – ATP	123 ± 1
– PEP	108 ± 9
– DTT	97 ± 7
– dATP, – dGTP, – dTTP	113 ± 3
– dATP, – dGTP, – dTTP, – dCTP	0.4 ± 0
[³ H]CDP tracer in	
complete NDP complement	100
– CTP, – GTP, – ATP	112 ± 2
– PEP	42 ± 2
– DTT	96 ± 16
– ADP, – GDP, – dTDP	114 ± 1
– ATP, – CTP, – GTP, – ADP, – GDP, – dTDP	102 ± 4
– ADP, – GDP, – dTDP, 0.04 mM CDP	49 ± 10
– ADP, – GDP, – dTDP, – CDP	0.02 ± 0.01

and 0.5 unit of alkaline phosphatase. The undigested material (containing 20–30% of the total radioactivity incorporated into acid-precipitable material) was removed by precipitation with ice-cold HClO₄ in the presence of 240 µg of carrier BSA and centrifugation (12800 *g*) at 4 °C for 15 s in a Beckman Microfuge. The digest of nucleic acids in the supernatant fraction was neutralized twice with 100 µl of 1 M-n-octylamine in Freon and analysed by h.p.l.c.

H.p.l.c. analysis of nucleic acid digest

A portion (50–100 µl) of nucleic acid digest was applied on to a 250 mm × 4.6 mm Alltech C₁₈ reverse-phase column and eluted with a linear gradient of 0.5–8% (v/v) acetonitrile in 10 mM-phosphate buffer, pH 3.6, containing 0.01% NaN₃, at a flow rate of 2 ml/min. Fractions (2 ml each) were collected, and their radioactivity was measured as described above; recovery of the radioactivity in eluted material was 90–98% of the applied digest.

Fig. 2. H.p.l.c. fractionation of the nucleic acid digest labelled with $[^3\text{H}]\text{CDP}$ (a) or $[^3\text{H}]\text{dCTP}$ (b) in lysoPtdCho-permeabilized S-49 cells

Permeabilized S-49 cells (3×10^6) were incubated for 45 min with $[^3\text{H}]\text{CDP}$ tracer in NDP reaction complement (a) or with $[^3\text{H}]\text{dCTP}$ tracer in dNTP reaction complement (b). Acid-precipitable material was degraded by sequential nuclease digestion, and the resulting acid-soluble hydrolysate was chromatographed on an Alltech C_{18} reverse-phase column. Fractions (2 ml each) were assayed for ^3H radioactivity (-----; shaded area); A_{254} (—) was monitored continuously.

RESULTS

Incorporation of cytosine nucleotides in permeabilized S-49 cells

Our first experiments were aimed at characterizing cytosine nucleotide incorporation into DNA of permeabilized S-49 cells under the conditions previously described by Reddy & Pardee (1982). Incorporation rates of $[^3\text{H}]\text{dCTP}$ and $[^3\text{H}]\text{CDP}$ were determined in permeabilized S-49 cells (Fig. 1). The NDP complement with $[^3\text{H}]\text{CDP}$ tracer was tested for its potential utilization for DNA via DNA replitase as proposed by Reddy & Pardee (1982). Fig. 1 illustrates that both tracers were readily incorporated, with comparable maximal rates of 2.2 and 2.9 pmol/min per 10^6 cells for dCTP and CDP respectively. These rates are equivalent to approx. 40% of the DNA-synthesis rate in the intact cells, the latter being calculated from the average DNA content per mouse lymphoblastoma cell of $7 \mu\text{g}$ of DNA/ 10^6 cells (Shapiro, 1970) and a 16 h doubling time in an exponential culture.

However, the co-substrate requirements for incorporation of either tracer into acid-precipitable macromolecules were disparate from those reported previously (Miller *et al.*, 1978; Reddy & Pardee, 1982) and expected for DNA synthesis. As Table 1 shows, most of the factors can be deleted from the reaction mixture without affecting the rate of incorporation of either tracer. Furthermore,

$[^3\text{H}]\text{dCTP}$ or $[^3\text{H}]\text{CDP}$ incorporation continued when all three complementary nucleotides were absent from the incubation mixture.

The acid-insoluble material was extracted from the cells incubated with cytosine nucleotide tracers, degraded with nucleases into nucleoside precursors and separated by h.p.l.c. (Figs. 2a and 2b). When $[^3\text{H}]\text{CDP}$ -labelled material was subjected to this procedure, the radioactivity was recovered quantitatively in the cytidine peak, with no label in the deoxycytidine fraction (Fig. 2a). Hence, incorporation of $[^3\text{H}]\text{CDP}$ via ribonucleotide reductase into DNA did not occur, in agreement with the results of Spyrou & Reichard (1983) but in contradiction with previous results (Reddy & Pardee, 1982). Further, even in the absence of cells, but in the presence of PEP and ATP, CDP was rapidly and quantitatively phosphorylated to CTP, which is very likely the proximate substrate that was utilized in the polymerization reaction. When $[^3\text{H}]\text{dCTP}$ was employed as the tracer with the permeabilized cells, radioactivity incorporated into cellular macromolecules was recovered in the fractions that are co-eluted with deoxycytidine (Fig. 2b).

Limited incorporation of purine nucleotides and dTTP in different cell lines

To answer the question as to whether the single nucleotide incorporation was a process specific to

Table 2. Substrate requirements for the incorporation of different tracers into macromolecules of permeabilized S-49 cells

The permeabilized cells ($3 \times 10^6/100 \mu\text{l}$ sample) were incubated for 20 min in complete reaction mixture containing 3 mM-ATP, 1.6 mM-DTT, four complementary nucleotides at 0.2 mM each (dNTP: dATP, dCTP, dGTP, dTTP; NTP: ATP, CTP, GTP, UTP; NDP: ADP, CDP, GDP, UDP; NMP: AMP, CMP, GMP, UMP) and appropriate tracer at $10 \mu\text{Ci/ml}$. In parallel samples, three of four complementary nucleotides were removed from the complete incubation mixture leaving only the tracer nucleotide at 0.2 mM and $10 \mu\text{Ci/ml}$. The tracer incorporation rate was calculated (mean $\pm$ range) from HClO_4 -insoluble radioactivity measurements in duplicate samples. [^3H]ATP incorporation was carried out at 1.2 mM-ATP and $20 \mu\text{Ci/ml}$ of the tracer to avoid excessive tracer dilution. The data are from a single experiment. Repeated experiments yielded similar results; averaged values (mean $\pm$ S.D., $n \geq 6$) of dNTP tracer incorporation in complete mixture at 37° are 2.52 ± 0.94 , 0.32 ± 0.19 and 0.17 ± 0.16 for [^3H]dCTP, [^3H]dGTP and [^3H]dTTP respectively.

Mixture	Tracer	Incorporation rate (pmol/min per 10^6 cells)		
		Complete		Tracer nucleotide only (37°C)
		0°C	37°C	
dNTP	[^3H]dCTP	0.04 ± 0.01	1.14 ± 0.10	1.15 ± 0.06
	[^3H]dGTP	0.14 ± 0.05	0.46 ± 0.26	0.19 ± 0.02
	[^3H]dTTP	0.04 ± 0.01	0.11 ± 0.01	0.08 ± 0.01
NTP	[^3H]ATP	0.01 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
	[^3H]GTP	0.09 ± 0.01	0.15 ± 0.01	0.28 ± 0.01
	[^3H]UTP	0.28 ± 0.01	1.24 ± 0.01	1.12 ± 0.09
NDP	[^3H]CDP	0.28 ± 0.01	1.05 ± 0.02	0.94 ± 0.01
	[^3H]GDP	0.04 ± 0.01	0.14 ± 0.01	0.10 ± 0.01
NMP	[^3H]AMP	0.11 ± 0.01	0.12 ± 0.01	0.11 ± 0.02
	[^3H]CMP	0.12 ± 0.02	0.92 ± 0.02	1.10 ± 0.12
	[^3H]GMP	0.29 ± 0.07	0.12 ± 0.01	0.20 ± 0.02
	[^3H]UMP	0.28 ± 0.01	0.58 ± 0.03	0.50 ± 0.03

cytosine precursors only, other nucleotide tracers were also incubated with the permeabilized S-49 cells. Only cytosine and uracil nucleotides were incorporated efficiently into acid-precipitable material (Table 2). In contrast, the permeabilized S-49 cells were incapable of significantly incorporating dTTP and purine nucleotides, regardless of their phosphorylation state, although all the nucleoside mono- and di-phosphates were metabolized by cellular enzymes to their corresponding nucleoside triphosphates (h.p.l.c. analysis of the cell-free incubation mixture; results not shown).

The incorporation rate of CDP or dCTP remained constant within the first 40 min of the incubation at 37°C (Figs. 3a and 3b). Decreasing the temperature to 0°C inhibited the incorporation into the non-specific level independent of the incubation time. With the use of guanine nucleotide tracers, permeabilized S-49 cells showed a rather low incorporation that was independent of the reaction temperature and length of incubation (Figs. 3a, 3b and 3c).

We also compared the incorporation rate of dCTP and dTTP in permeabilized cells of different origin, tumorigenesis and growth characteristics, namely mouse lymphoblastoma S-49, mouse neuroblastoma $\times$ rat glioma NG 108-15 hybrid cells and Abelson-leukaemia-virus-transformed mouse macrophages RAW 264.7 (Table 3). In a qualitative sense, all three cell lines showed a similar trend of a selective dCTP incorporation, with no stringent requirement for all four complementary nucleotides. However, dCTP incorporation was surprisingly high in the neuroblastoma hybrid cells. This is not a specific characteristic of neuroblastoma cells in general, as another neuroblastoma line tested (human SK-N-SH; Biedler *et al.*, 1973) failed to show elevated dCTP incorporation (results not shown).

The incorporation characteristics of three dNTP tracers ([^3H]dCTP, [^3H]dGTP and [^3H]dTTP) remained unchanged when the reaction was carried out in a modified buffer as described for DNA synthesis in lysoPtdCho-permeabilized cells by Miller *et al.* (1978). An alternative permeabilizing procedure with hypotonic buffer containing 30 mM-2-mercaptoethanol (Berger & Johnson, 1976) also failed to cause any significant changes in the incorporation pattern of deoxyribonucleotides (results not shown).

We further tested the possibility that residual endogenous pools of nucleotides in the permeabilized cells might be preferentially used for DNA synthesis, thereby preventing dNTP tracers being incorporated into DNA. However, the permeabilized cells depleted of deoxyribonucleotides with the addition of 2 mM-hydroxyurea (Thelander & Reichard, 1979) or of their guanine nucleotides by 2 μM -mycophenolic acid (Cohen *et al.*, 1981) failed to increase the incorporation of exogenously supplied [^3H]dGTP. Thus we had to exclude the possibility that residual cellular pools of purine nucleotides and dTTP serve as potential co-substrates for any DNA synthesis occurring in permeabilized S-49 cells.

DNA synthesis in permeabilized or homogenized cells supplemented with DNA template

Comparison of the incorporation of [^3H]dCTP and [^3H]dTTP in lysoPtdCho-permeabilized and/or homogenized S-49 cells (Table 4) showed that the incorporation of deoxyribonucleotides was not restricted by subcellular structures (e.g. nuclear membrane) in the permeabilized cells. Homogenization of the permeabilized cells decreased the incorporation of [^3H]dCTP, whereas [^3H]dTTP uptake into acid-precipitable material remained unchanged. Further, the procedure of cell permeabilization

Fig. 3. Incorporation of the nucleotide tracers into macromolecules of the lysoPtdCho-permeabilized S-49 cells

Permeabilized cells ($3 \times 10^6/100 \mu\text{l}$) were incubated with all four complementary nucleotides and the radioactive tracer at $10 \mu\text{Ci/ml}$ at 37°C (closed symbols) or 0°C (open symbols). (a) dNTP reaction complement with [^3H]dCTP (●, ○) or [^3H]dGTP (■, □) tracer; (b) NDP reaction complement with [^3H]CDP (●, □) or [^3H]GDP (■, □) tracer; (c) NTP reaction complement with [^3H]GTP tracer. The tracer incorporation into cellular macromolecules was calculated from the HClO_4 -insoluble radioactivity measured in duplicate samples.

could not account for the impaired DNA synthesis, since homogenates of intact cells were also defective in incorporating dTTP.

However, DNA synthesis in permeabilized or homogenized cells was initiated upon the addition of denatured salmon testes DNA (Table 4). The presence of heat-denatured DNA template led to a significant increase of dTTP incorporation that was dependent on the presence of all four complementary nucleotides. In contrast, the incorporation of dCTP proceeded at the same rate in the presence or absence of the complementary nucleotides. The incorporation of both dCTP and dTTP in the presence of extracellular DNA matrix was approx. 2-fold higher in permeabilized than in homogenized cells.

The tracer fraction in alkali-resistant macromolecules (e.g. DNA) is given in parentheses in Table 4. It amounted to only 20% of [^3H]dCTP total incorporation in the permeabilized cells and increased to 40% in the presence of denatured salmon testes DNA in either permeabilized cells or cellular homogenates. On the other hand, 80% of [^3H]dTTP radioactivity that was incorporated into the extracellular matrix was resistant to alkali, as expected for tracer incorporated into DNA.

Tracer incorporation into S-49 DNA by labelling before permeabilization

S-49 cells were preincubated with [^3H]dT at 0°C in order to label the endogenous dTTP pool in the absence of DNA synthesis. After cell permeabilization and resuspension in 37°C buffer, this endogenous [^3H]dTTP was slowly incorporated into DNA over ~ 10 min, after which time incorporation virtually ceased (Fig. 4a), although [^3H]dTTP concentration in the incubation mixture, when measured by h.p.l.c., was not decreased for at least 30 min. If one treats cells in an identical fashion but adds [^3H]dTTP tracer to the medium after cell permeabilization (Fig. 4b), there was no difference to the results obtained with prior [^3H]dT labelling. Therefore [^3H]dTTP generated in intact cells gave rise to an incorporation profile similar to that given by the [^3H]dTTP tracer added exogenously to the permeabilized cells. The cessation of DNA synthesis after 10–15 min was not the result of cell permeabilization, because the

Table 3. Substrate requirements for dCTP and dTTP incorporation into macromolecules of different cell lines after permeabilization with lysoPtdCho

The rate of [^3H]dCTP or [^3H]dTTP incorporation was calculated (mean $\pm$ s.d.) from HClO_4 -insoluble radioactivity measurements in duplicate samples of the permeabilized cells incubated for 30 min with 3 mM-ATP, 0.04 mM-CTP, 0.04 mM-GTP, 10 mM-PEP, 8 mM-DTT, all four dNTPs (dATP, dCTP, dGTP and dTTP) at 0.2 mM each and the radioactive tracer at $10 \mu\text{Ci/ml}$ in the complete reaction mixture. In the other set of samples three of four complementary deoxyribonucleotides were omitted, except for the tracer nucleotide present at 0.2 mM and $10 \mu\text{Ci}$. The numbers of independent repeat experiments (n) are given.

Tracer	Cell line	n	Incorporation rate (pmol/min per 10^6 cells)		
			Complete		Tracer nucleotide only (37°C)
			0°C	37°C	
[^3H]dCTP	S-49	12	0.13 ± 0.08	2.5 ± 0.9	2.2 ± 0.9
	NG-108	4	0.74 ± 0.06	26.0 ± 3.0	29.3 ± 4.6
	RAW 264.7	2	0.15 ± 0.02	1.7 ± 0.3	2.5 ± 0.7
[^3H]dTTP	S-49	6	0.05 ± 0.02	0.17 ± 0.16	0.16 ± 0.09
	RAW 264.7	2	0.14 ± 0.01	0.13 ± 0.01	0.14 ± 0.01

Fig. 4. Incorporation of endogenous (a) or exogenous (b) $[^3\text{H}]\text{dTTP}$ into intracellular DNA in intact or permeabilized S-49 cells

(a) S-49 cells were prelabelled with $[^3\text{H}]\text{dT}$ tracer at 4°C for 30 min before permeabilization (for incubation conditions, see the Experimental section). Whereas $[^3\text{H}]\text{thymine}$ nucleotides accumulated in the cells, tracer incorporation into DNA was minimal. Then the extracellular tracer was washed out with ice-cold medium, the cell pellet was either permeabilized with lysoPtdCho (permeabilized cells) or was kept untreated (intact cells) and the resulting cells were resuspended in buffer and incubated at 37°C to determine ^3H incorporation into DNA. The attained $[^3\text{H}]\text{dTTP}$ concentration of 6×10^5 d.p.m./ 5×10^6 cells did not measurably decline during the 30 min incubations. The higher level of tracer incorporation into DNA of the intact cells results from a lack of nucleotide pool dilution in permeabilized cells by the resuspension medium. (b) Cells were treated as described for (a), except that the tracers were added after resuspension of the resulting cell pellets at 37°C . Intact cells were labelled with $[^3\text{H}]\text{dT}$, whereas permeabilized cells were labelled with $[^3\text{H}]\text{dTTP}$, the latter at a concentration identical with that achieved by prelabelling the cells with $[^3\text{H}]\text{dT}$ at 0°C (see a).

same pattern of DNA synthesis was observed with intact cells labelled at 37°C with $[^3\text{H}]\text{dT}$ under otherwise identical conditions (Figs. 4a and 4b). As observed in the above experiments, the DNA synthesis in permeabilized cells (both prelabelled with $[^3\text{H}]\text{dT}$ or continuously labelled at 37°C with $[^3\text{H}]\text{dTTP}$) was greatly stimulated by addition of denatured salmon testes DNA and continued at a constant 5-times-higher rate for at least 30 min (results not shown).

DISCUSSION

With the use of three different cell lines, two methods of cell permeabilization and, alternatively, cell homogenization, we demonstrated that only cytosine and uracil nucleotides were efficiently incorporated into nucleic acids. Unlike DNA-template-directed nucleic acid synthesis, the incorporation of cytosine and uracil nucleotides was independent of the presence of the three other complementary nucleotides.

The incorporation that proceeded in the presence of single tracer nucleotide seems to be a DNA-matrix-independent process that resembles that catalysed by

terminal nucleotidyltransferase(s). The existence of at least two enzymes capable of mediating the latter process was documented by Kato *et al.* (1967) and by Gottesman & Canellakis (1966). The enzyme described by the former group of researchers is recently again a subject of a considerable interest because of its potential as a cancer marker in certain types of leukaemias and as an indicator of lymphocyte differentiation (Bollum, 1982). However, the product of intracellular polymerization of cytosine nucleotides does not share the features of substrate specificity and resistance to alkaline hydrolysis that were ascribed to the polynucleotide material formed with terminal nucleotidyltransferases. These differences might reflect the fact that the enzymic activities were characterized as the purified preparations of cytoplasmic (Kato *et al.*, 1967) or nuclear (Gottesman & Canellakis, 1966) origin, whereas in the permeable or homogenized cells the enzyme might be affected by other cellular components. Alkali-lability of the dCTP polymerization product formed in permeabilized and homogenized S-49 cells (Table 4) suggests a rather short deoxyribonucleotide sequence attached to an alkali-sensitive region of the macromolecule, whereas replication leading to alkali-

Table 4. Substrate and extracellular DNA matrix requirements for [³H]dCTP and [³H]dTTP incorporation into macromolecules of lysoPtdCho-permeabilized or homogenized S-49 cells

The permeabilized or homogenized S-49 cells were incubated for 45 min in the complete incubation mixture consisting of 3 mM-ATP, 0.04 mM-CTP, 0.04 mM-GTP, 10 mM-PEP, 5 mM-DTT, all four deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, dTTP) at 0.2 mM each and the radioactive tracer at 20 μ Ci/ml, or in the complete mixture depleted of three of four complementary dNTPs with appropriate tracer only at 0.2 mM and 20 μ Ci/ml. Before incubation cells were (1) permeabilized with lysoPtdCho, (2) homogenized, (3) supplemented in the reaction mixture with 10 μ g of heat-denatured salmon testes DNA or (4) exposed in the reaction mixture to 40 μ g of lysoPtdCho/ml. The total acid-precipitable radioactivity and alkali-resistant fraction (in parentheses) were determined in duplicate samples and the incorporation rate of the tracer was calculated (mean $\pm$ range). The data are from a single experiment; independent repeat experiments yielded similar results.

Temperature (°C)	Cell preparation				Incorporation rate (pmol/min per 10 ⁶ cells) (% alkali-resistant)			
					[³ H]dCTP		[³ H]dTTP	
	1	2	3	4	Four dNTPs	dCTP only	Four dNTPs	dTTP only
0	1				0.19 $\pm$ 0.07 (15 $\pm$ 0)	0.06 $\pm$ 0.01 (16 $\pm$ 3)		
37	1				3.0 $\pm$ 0.1 (21 $\pm$ 4)	2.8 $\pm$ 0.2 (18 $\pm$ 0)	0.18 $\pm$ 0.08 (55 $\pm$ 11)	0.13 $\pm$ 0.03 (51 $\pm$ 5)
	1	2			0.56 $\pm$ 0.03 (22 $\pm$ 1)	0.67 $\pm$ 0.01 (15 $\pm$ 0)	0.07 $\pm$ 0.01 (67 $\pm$ 16)	0.08 $\pm$ 0.01 (49 $\pm$ 11)
		2			0.99 $\pm$ 0.06 (15 $\pm$ 3)	0.76 $\pm$ 0.02 (11 $\pm$ 1)	0.21 $\pm$ 0.05 (59 $\pm$ 3)	0.07 $\pm$ 0.01 (36 $\pm$ 1)
	1		3		3.5 $\pm$ 0.2 (41 $\pm$ 0)	3.5 $\pm$ 0.5 (23 $\pm$ 2)	2.1 $\pm$ 0.2 (85 $\pm$ 0)	0.68 $\pm$ 0.07 (76 $\pm$ 1)
	1	2	3		1.6 $\pm$ 0.1 (92 $\pm$ 0)	0.90 $\pm$ 0.09 (22 $\pm$ 0)	1.1 $\pm$ 0.1 (80 $\pm$ 1)	0.23 $\pm$ 0.11 (48 $\pm$ 20)
		2	3		1.4 $\pm$ 0.2 (42 $\pm$ 1)	1.3 $\pm$ 0.3 (23 $\pm$ 0)	1.2 $\pm$ 0.2 (81 $\pm$ 0)	0.20 $\pm$ 0.03 (62 $\pm$ 1)
		2	3	4	1.3 $\pm$ 0.2 (47 $\pm$ 1)	0.80 $\pm$ 0.06 (27 $\pm$ 1)	0.99 $\pm$ 0.20 (79 $\pm$ 1)	0.20 $\pm$ 0.01 (59 $\pm$ 1)

resistant DNA proceeds concomitantly when extracellular DNA template and all four deoxyribonucleotides are accessible (Table 4).

Two relevant reports exist on single nucleotide incorporation in systems *in vitro* optimized for DNA synthesis. During replication *in vitro* of simian-virus-40 DNA in permeabilized cells (Oda *et al.*, 1981), deletion of dATP, dCTP and dGTP only partially decreased the rate of dTTP incorporation, providing that ribonucleoside triphosphates were present in the reaction mixture. A purified nuclear DNA polymerase from HeLa cells was also able to polymerize a single dNTP in the absence of any other ribo- and deoxyribo-nucleoside triphosphate (Schlabach *et al.*, 1971).

The low incorporation rate of purine nucleotides and dTTP in permeabilized or homogenized cells cannot be explained by residual sources of these substrates for DNA synthesis or by limited penetration of dNTPs through cytoplasmic or nuclear membrane in the permeabilized cells. The restoration of the ability of S-49 cells to incorporate all of the constituent deoxyribonucleotides into DNA upon the addition of a denatured DNA implies that intracellular DNA present in the permeabilized or homogenized S-49 cells did not serve as an efficient matrix in DNA synthesis *in vitro*. The results of prelabelling at 4 °C with [³H]dT or subsequent continuous labelling at 37 °C with [³H]dT or [³H]dTTP (Fig. 4) show that, under the applied conditions *in vitro*, DNA-matrix activity is suppressed in permeabilized and intact cells as well. The factors involved could be long exposure of the cells to low temperature, too high cell

density or, finally, inadequate buffer, lacking some of the factors necessary for DNA replication. Because DNA synthesis continued for a short period of time after placing the cells at 37 °C and then ceased, one can propose that chain initiation rather than elongation is impaired under these conditions.

As an alternative explanation for the low DNA synthesis rate *in vitro* one can consider that extracellular deoxyribonucleoside triphosphates may not be available as a substrate in the polymerization process. It was postulated that a multienzyme replicative complex synthesizes deoxyribonucleoside triphosphates, the ultimate substrates for DNA polymerase, for its own use, without equilibration of the DNA-precursor pool with the general cellular nucleotide pools (Taheri *et al.*, 1981; Wickremasinghe *et al.*, 1982; Reddy & Pardee, 1982). Thus the exogenously supplied nucleotides could be prevented from being incorporated into the small, 'replicative', pool. This view is consistent with recent results from this laboratory showing the selective utilization in the DNA synthesis of the dGTP pool generated from a 'de novo' pathway via ribonucleotide reductase over the cellular dGTP pool generated by the salvage pathway from dG via kinases (Nguyen & Sadée, 1986). However, we were unable to observe any significant difference in the [³H]dTTP incorporation into endogenous DNA of permeabilized cells, whether the [³H]dTTP pool was derived from [³H]dT before permeabilization (thus labelling an endogenous pool with direct access to the DNA-replication fork) or added as [³H]dTTP after permeabilization. These results do not

rule out compartmentation or channelling, because of the lag time between [³H]dT labelling, cell permeabilization and DNA incorporation with possible equilibration between subcellular nucleotide pools. It appears that permeabilized cells are unsuitable for investigating these phenomena. Hence previous evidence on nucleic-acid-precursor channelling that was derived from permeabilized mammalian cells has to be critically reassessed in view of the demonstration here that nucleotide incorporation into the acid-insoluble macromolecules is not representative of template-dependent nucleic acid synthesis.

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