Incorporation of Deoxynucleotides into DNA by Diethylaminoethyldextran-Treated Lymphocytes†

Richard M. Fox,‡ Jean F. Mynderse, and Mehran Goulian*

ABSTRACT: In the presence of diethylaminoethyldextran cultured human lymphocytes will utilize deoxynucleotides for the synthesis of DNA, whereas in the absence of diethylaminoethyldextran no incorporation of deoxynucleotides is detected. Labeled deoxynucleoside mono-, di-, and triphosphates are incorporated into DNA at approximately the same rate. Deoxynucleotide incorporation is essentially linear for 10 min but continues at a gradually diminishing rate for an additional 20 to 50 min. The initial rate of DNA synthesis is at least 20 to 40% of the in vivo rate, and in those cells that are in S phase 0.7–1.5% of the DNA is synthesized. By the three properties examined (restriction to S phase, semiconservative mode, and initial product in short chains), DNA synthesis in diethylaminoethyldextran-treated cells resembles DNA synthesis in vivo.

Studies on the mechanism of DNA synthesis in intact animal cells have been hindered by the impermeability of the cell membrane to nucleotide precursors of DNA. With microorganisms this problem has been partly overcome by treatment of cells with organic solvents, such as toluene (Moses and Richardson, 1970; Matsushita et al., 1971) and ether (Vosberg and Hoffmann-Berling, 1971; Howell and Walker, 1972), or detergents (Ganesan, 1971; Hereford and Hartwell, 1972), or by plasmolysis (Gros et al., 1967; Wickner and Hurwitz, 1972). Cells so treated become permeable to nucleotides, including dNTPs, and this has permitted in vitro studies on DNA synthesis (Goulian, 1971; Klein and Bonhoeffer, 1972; Gefter, 1975).

In most studies on animal cells, broken or fractionated cell preparations have been used, with disruption of the structural and functional integrity of the cell (Friedman and Mueller, 1968; Winnaker et al., 1971; Burgoyne, 1972; Kidwell, 1972; Lynch et al., 1972; Probst et al., 1972; Hershey et al., 1973; Lazarus, 1973; Hallick and Namba, 1974; Hunter and Francke, 1974; Tseng and Goulian, 1975a). Recently, two reports have appeared describing permeabilization of animal cells by treatment with hypotonic buffer (Seki et al., 1975) or cold shock (Berger and Johnson, 1976). We describe here another method for direct incorporation of deoxynucleotides into DNA of nondisrupted animal cells. Entry of nucleotide into the cells takes place in the presence of DEAE[1]-dextran, a polycationic polysaccharide derivative, which has been used to potentiate uptake of nucleic acids into cultured animal cells (Pagano, 1970). The conditions for incorporation of nucleotide are described, together with properties of the system.

Experimental Procedure

Reagents. Unlabeled nucleotides were from P-L Biochemicals and Terramarine. ^{3}H-labeled nucleosides and nucleotides and [^{14}C]dThd were purchased from Schwarz/Mann. All ^{3}H-labeled nucleotides were purified by paper chromatography (see below) before use. α-^{32}P derivatives of dTTP, dTDP, and dTMP were synthesized with *E. coli* thymidine kinase (Okazaki and Kornberg, 1964) and [γ-^{32}P]ATP (Glynn and Chapell, 1964). For the preparation of [α-^{32}P]dGTP, 3′-[5′-^{32}P]dGDP was made from 3′-dGMP using T4 polynucleotide kinase (Richardson, 1965) and [γ-^{32}P]ATP, followed by removal of the 3′-P with T4 3′-phosphatase (Becker and Hurwitz, 1967). BrdUTP was synthesized by the method of Bessman et al. (1958). DEAE-dextran, size approximately 5 × 10^5 daltons, was purchased from Pharmacia. Pronase was obtained from Calbiochem, hydroxyurea from Nutritional Biochemical, and sarkosyl from Geigy.

Cells. A permanent human lymphoblastoid cell line ("8866") derived from normal splenic lymphocytes was kindly provided by Dr. Richard Lerner. The cells were maintained in the log phase of growth (3–10 × 10^5 cells/mL) in a modified Eagle's medium (Flow Auto-Pow MEM) containing 10% fetal calf serum in suspension culture at 37 °C, with a doubling time of 15 h. Cells were harvested at a cell concentration of 6–8 × 10^5/mL by centrifugation (130*g*, 10 min, 4 °C) and washed once in ice-cold 60 mM Tris-HCl, pH 7.8, 120 mM NaCl, 5 mM sodium phosphate, pH 7.8, 5 mM $MgCl_2$, 10 mM glucose, 0.2 mM dithiothreitol (buffer A); they were then resuspended in buffer A at a concentration of 8 × 10^7/mL, and immediately used in the incubation.

Conditions for Incubation with DEAE-dextran. Unless stated otherwise, the incubation mixture had a volume of 0.3 mL and contained 60 mM Tris-HCl, pH 7.8, 60 mM NaCl, 5 mM sodium phosphate, pH 7.8, 0.5 mM each of dCTP, dATP, dGTP, and [^{3}H]dTTP (30–100 cpm/pmol), 2.5 mM ATP, 20 mM trisodium phosphoenolpyruvate, 5 mM $MgCl_2$, 10 mM glucose, 0.2 mM dithiothreitol, 150 μg/mL DEAE-dextran, and cells at 1.3 × 10^7/mL. The mixture was incubated at 37 °C in a shaking water bath (150 rpm).

Estimation of Label Incorporation into DNA. With most incubation mixtures, which did not contain ^{3}H-labeled purines, the reaction was terminated by chilling (0 °C) followed by the addition of 1 mL of a lysis mixture containing EDTA (0.01 M), NaOH (1 M), potassium pyrophosphate (0.05 M), and sodium tripolyphosphate (0.05 M). This was heated (100 °C, 1 min) and then rechilled; carrier herring sperm DNA (250 μg) was added followed by 2 mL of 2 M HCl and, after 5 min at 0 °C, the precipitate was collected by centrifugation (12 000*g*, 10

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‡ Present address: Ludwig Institute for Cancer Research, Sydney Cancer Therapy Unit, University of Sydney, Sydney N.S.W. 2006, Australia.

[1] Abbreviations used are: BrdUTP, bromodeoxyuridine triphosphate; sarkosyl, sodium dodecyl sarcosinate; DEAE, diethylaminoethyl; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride; EDTA, (ethylenedinitrilo)tetraacetic acid.

min). The precipitate was redissolved in 1 mL of the lysis mixture (see above) with heating (100 °C, 1 min), reprecipitated with 2 mL of 2 M HCl (0 °C, 5 min), and collected by filtration onto glass-fiber disks (Whatman GF/C). The disks were washed extensively, first with cold 0.01 M HCl and then acetone, and finally dried and counted in a toluene mixture with a liquid scintillation counter (30% efficiency).

In experiments that included ^{3}H-labeled purines a nonalkaline procedure was followed, since alkali, especially with heat, causes exchange of the [^{3}H]purine with H of water, with consequent loss of the label. The reaction was stopped by chilling in ice water followed by separation of cells by centrifugation (12 000*g*, 10 min) (tests of the procedure showed that there is no detectable incorporation of precursors into DNA at 0 °C for at least 60 min). The cells were resuspended in 0.2 mL of buffer A, lysed by addition of 0.2 mL of a mixture of EDTA (0.1 M), sarkosyl (3%), and Pronase (2 mg/mL), and heated at 60 °C for 90 min. The Pronase-digested lysate was chilled and to it was added 15 mL of a mixture of potassium pyrophosphate (0.1 M) and sodium tripolyphosphate (0.1 M), followed by 5 mL of 4 M HCl; the remaining steps for filtration and counting on glass-fiber disks were as described above. It was found that the presence of sarkosyl can result in incomplete precipitation of nucleic acids; this was prevented by adding 2 volumes of ethanol to the acid precipitation mixture prior to filtration.

In experiments intended to show that the substrate label was incorporated specifically into nuclear DNA, the reaction was stopped by chilling and the cells were collected by centrifugation and resuspended at 8×10^7/mL in buffer A containing 10% sucrose. Nonidet P-40 (Shell) was added to a final concentration of 0.25% to lyse plasma membranes but leave nuclei intact. After 5 min at 0 °C, the nuclei were collected by centrifugation, washed twice with buffer A containing sucrose, followed by determination of label incorporation into DNA as described above.

Autoradiographic Analysis. Cells were partially synchronized by 15-h incubation with 2.5 mM dThd, followed by resuspension in fresh medium without dThd for 1 h. Incubations were carried out as described above using [^{3}H]dTTP at 15 mCi/μmol, or [^{3}H]dThd (0.36 mCi/μmol; 2 μCi/0.3 mL of incubation) in place of dTTP, with and without DEAE-dextran, or both [^{3}H]dThd and [^{3}H]dTTP in the presence of DEAE-dextran. After incubation for 60 min, the cells were washed, spread on slides, coated with emulsion (Ilford L4), and exposed for 52 days at 4 °C. The emulsion was then developed, the cells were stained with Giemsa, and 500 cells were counted and scored for the presence or the absence of nuclear labeling. Labeling with [^{3}H]dTTP alone was somewhat lighter than with [^{3}H]dThd, possibly explaining the slightly lower proportion of labeled cells with [^{3}H]dTTP alone (see below).

Analysis of [^{3}H]dThd-Labeled Intracellular Nucleotide Pools. [^{3}H]dThd (10 μCi/mL, 53 mCi/μmol) was added to growing lymphocytes 1 h before harvesting. The standard procedure for incubation with DEAE-dextran was followed except for an additional wash of the cells with fresh culture medium (prior to incubation) to remove excess [^{3}H]dThd. After incubation, the cells were collected by centrifugation (400*g*, 10 min) and extracted by stirring with 60% methanol (0.25 mL/10^6 cells) at 0 °C for 30 min; following this, solid material was removed by centrifugation and the extract was dried under reduced pressure. The residue, taken up in a small volume of water, as well as portions of the incubation medium, were assayed for total radioactivity and analyzed for the distribution of radioactivity in dThd nucleotides by electrophoresis in citrate buffer (see below).

Sedimentation Velocity Analysis of DNA. A culture of lymphocytes was labeled by growth for 16 h in the presence of 1 nCi/mL [^{14}C]dThd (0.33 mCi/mmol). In vivo pulse labeling was begun by the addition of 20 μCi/mL [^{3}H]dThd (55 mCi/μmol); samples were removed at 20 s and at 1, 3, 10, and 30 min, and added immediately to an equal volume of ice-cold ethanol. The 20-s sample size was 10 mL and contained 8×10^6 cells; the subsequent four samples were 2 mL but sufficient unlabeled cells diluted in ethanol were added to give each an amount of DNA equivalent to the 20-s sample.

A second portion of [^{14}C]dThd-labeled cells was incubated for the same five periods, under standard conditions with DEAE-dextran, except that the specific activity of [^{3}H]dTTP was varied as follows: 970, 360, 140, 37, and 37 cpm/pmol for the 20-s and 1-, 3-, 10-, and 20-min incubations, respectively. Each incubation (0.6 mL) contained 8×10^6 cells, and the reaction was terminated by adding an equal volume of ice-cold ethanol.

The cells from both in vivo and in vitro labeled preparations were collected by centrifugation (12 000*g*, 10 min), and resuspended in 0.5 mL of 50 mM Tris-HCl, pH 8.1, 140 mM NaCl, 50 mM EDTA. Pronase, followed by sarkosyl, was added to each sample, at final concentrations of 1 mg/mL and 1%, respectively. After incubation for 18 h at 37 °C, the mixture was made 0.3 M in KOH and layered onto a 31-mL sucrose (5–20%) gradient in 0.3 M KOH, 0.7 M KCl, 1 mM EDTA, which was over a 6-mL shelf of 60% sucrose in the same solution. The gradients were centrifuged in a Spinco SW 27 rotor at 25 000 rpm for 15 h at 4 °C. Fractions (1.5 mL) were collected from the bottom and to each was added 0.1 mL of a mixture of carrier herring sperm DNA (2.5 mg/mL), potassium pyrophosphate (0.1 M), and sodium tripolyphosphate (0.1 M). The DNA was precipitated by the addition of 1.5 mL of 2 M HCl, and was collected and counted on glass-fiber disks as described above. The top six fractions of each gradient also received 6 mL of ethanol in addition to the HCl to prevent interference with precipitation of DNA caused by sarkosyl in the sample (see above). ^{3}H counts were corrected for ^{14}C overlap before plotting in the figures, and background was subtracted for the in vitro data (DEAE-dextran incubations), which are presented as "pmol of dTMP"; background was not subtracted for the in vivo data.

Equilibrium Density Gradient Centrifugation. Lymphocytes were labeled by growth for 20 h with 1 nCi/mL [^{14}C]-dThd (0.33 mCi/mmol). A preincubation mixture with DEAE-dextran (1.0 mL) was prepared as described above for the normal incubation, except for inclusion of BrdUTP (0.5 mM) and omission of all other dNTPs. After 10 min at 37 °C, 0.5 mM each of dATP, dGTP, and [^{3}H]dCTP (200 cpm/pmol) were added and the mixture was incubated for an additional 10 min. Incorporation was 10.1 pmol of dCMP/10^6 cells in 10 min. If the preincubation with BrdUTP was omitted, a range of hybrid densities resulted, indicating incomplete replacement of the cellular dTTP pool by BrdUTP from the incubation mixture. The incubation was terminated by the addition of 1.7 mL of buffer A and 2 mL of 2% sodium dodecyl sulfate. The mixture was sheared by ten passages through a 26-gauge needle; 0.4 mL of 0.5 M EDTA (Na^+) (pH 7.6) was added and the mixture was incubated for 18 h at 37 °C with Pronase (300 μg/mL). An aliquot (0.2 mL) was saved for the determination of total incorporation and velocity sedimentation analysis (below); to the remainder was added CsCl to a density of 1.745 g/cm^3, and the mixture (6.7 mL) was centrifuged in a Spinco 50 rotor at 44 000 rpm for 48 h at 20 °C. Fractions were collected from the bottom of the gradient, and aliquots (0.20 of each fraction) were taken for precipitation with acid

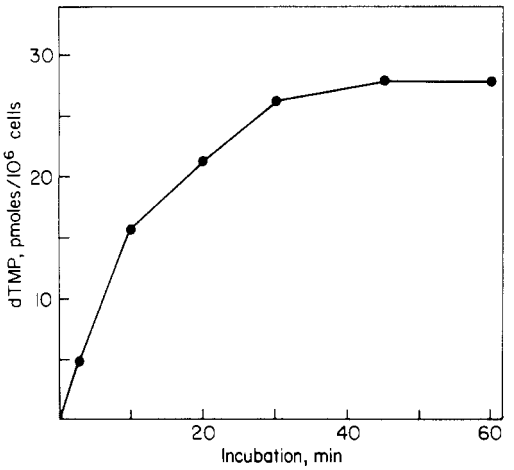

FIGURE 1: Incorporation of label from [^{3}H]dTTP into lymphocytes in the presence of DEAE-dextran. The incubation was carried out under standard conditions (Experimental Procedure).

TABLE I: Requirements for DNA Synthesis in Lymphocytes in the Presence of DEAE-dextran.[a]

Incubation conditions	Incorp rate (pmol of dTMP min^{-1} (10^6 cells)$^{-1}$)
Complete mixture	3.46
−DEAE-dextran	0.26
−ATP	2.94
−Phosphoenolpyruvate (+60 mM NaCl)[b]	2.86
−ATP, −phosphoenolpyruvate (+60 mM NaCl)[b]	2.20
−Glucose	2.54
−NaCl	3.42
−NaCl, +KCl (60 mM)	2.14
−Dithiothreitol	3.58
+*N*-Ethylmaleimide (1 mM)	0.06
+GTP, CTP, UTP (each, 0.5 mM)	3.10
+Ado, Cyd, Guo, and Urd (each, 0.1 mM)	3.10

[a] Conditions for incubations (5 min) and measurement of incorporation into DNA are described in Experimental Procedure, with changes as noted. [b] NaCl is added to replace Na^+ normally included as trisodium phosphoenolpyruvate.

and collection on glass-fiber disks for counting, as described above.

Size of the sheared DNA was estimated by velocity sedimentation of a portion (0.1 mL) in a 4-mL neutral sucrose gradient (1 M NaCl, 0.01 M Tris-HCl, pH 8.0, 1 mM EDTA) in the Spinco SW 56 rotor at 45 000 rpm for 4 h at 20 °C; a 12S marker of minicircular DNA from *E. coli* 15T (Cozzarelli et al., 1968) was present in a separate tube. The sheared fragments had a distribution from 12 to 19 S, with an average size of 15 S, which is equivalent to 2.7×10^6 daltons (Studier, 1965).

Analysis of Nucleotides in the Incubation Medium. Aliquots from the incubation mixtures were chilled (0 °C) and centrifuged (400*g*, 10 min) to remove cells, and to the supernatant was added 0.05 volume of 1 M EDTA (Na^+) (pH 7.6).

Thin-layer chromatography was on poly(ethyleneimine) cellulose thin-layer plates (Brinkman), with development in 1.0 M LiCl.

Paper chromatography was carried out on Whatman 3MM paper in isobutyric acid, water, concentrated NH_3, 66:33:1 (v/v).

Electrophoresis was on Whatman 3MM paper at 50–75 V/cm using 0.02 M sodium citrate buffer, pH 5.0.

Results

Incorporation of Labeled Deoxynucleotide into Lymphocyte DNA in the Presence of DEAE-dextran. In a randomly growing population of cultured human lymphocytes, which have a doubling time of 15 h, the overall rate of DNA synthesis is 28 pmol of nucleotide min^{-1} (10^6 cells)$^{-1}$ (Tseng and Goulian, 1975a). When the cells are incubated in a buffered salt solution in the presence of the four dNTPs, ATP, phosphoenolpyruvate, and DEAE-dextran, incorporation of the ^{3}H label from dTTP indicates an initial rate of DNA synthesis that is 20–40% of the in vivo rate (1.5–3.5 pmol of dTMP min^{-1} (10^6 cells)$^{-1}$) (Figure 1). There may be some underestimation of initial rate due to dilution of the label by cellular nucleotide pools (see below). The rate of incorporation into DNA appears linear for approximately 10 min, but thereafter gradually diminishes until by 30 to 60 min it has essentially stopped. To examine the relationship of synthesis in vitro with DEAE-dextran and dNTPs to normal DNA synthesis in vivo, the radioautographic labeling pattern was compared for cells labeled with [^{3}H]dThd and/or [^{3}H]dTTP (+ DEAE-dextran). The proportion of cells labeled with [^{3}H]dThd in the absence or presence of DEAE-dextran was 50 and 51%, respectively, with [^{3}H]dTTP plus DEAE-dextran 45% were labeled, and when both [^{3}H]dThd and [^{3}H]dTTP were present (plus DEAE-dextran) the figure was 54% (see Experimental Procedure). This result indicates that the synthesis in vitro was restricted to S phase cells. A maximum of 0.7–1.5% of the DNA is synthesized in vitro in S phase cells (Figure 1).

The initial rate of label incorporation is the same whether measured with [^{3}H]dTTP (Figure 1) or [α-^{32}P]dTTP. After 10 min of incubation, the rate in some experiments is slightly higher with [^{3}H]dTTP than with [α-^{32}P]dTTP, for reasons that are not clear (the difference is not due to a fall in specific activity resulting from loss of the α-^{32}P label, since this was less than 2%).

Activity is reduced by approximately $\frac{1}{6}$ when either ATP or phosphoenolpyruvate is omitted, and by $\frac{1}{3}$ when both are absent (Table I). NaCl is not needed in the incubation mixture for optimal activity, but replacement by KCl results in inhibition. Dithiothreitol also is not essential, but *N*-ethylmaleimide eliminates almost all activity. There is no augmentation of activity by inclusion of ribonucleosides or GTP, UTP, and CTP.

Incorporation of label from [^{3}H]dTTP falls by greater than 90% if DEAE-dextran is omitted (Table I and Figure 2). A much higher (and variable) residual activity, in the absence of DEAE-dextran, results with most preparations of commercial [^{3}H]dTTP if they are not first purified. This may be due to contaminating [^{3}H]dThd, which readily enters untreated cells. The low residual incorporation with purified dTTP without DEAE-dextran may also be due to dThd formed during the incubation, for no incorporation is detected without DEAE-dextran if α-^{32}P-labeled dNTP is used as substrate (see below). The optimal concentration for DEAE-dextran is influenced by cell concentration, but the relationship is not simply stoichiometric (Figure 2). At the usual concentration of $1.2–1.4 \times 10^7$ cells/mL the activity is greatest with DEAE-dextran at 100–200 μg/mL.

Highest rates of DNA synthesis are at pH 7.8; activity is reduced to 82% at pH 8.4, and to 77% at pH 7.3. Maximal activity results with Mg^{2+} at 5 mM; the rate is reduced to 89%

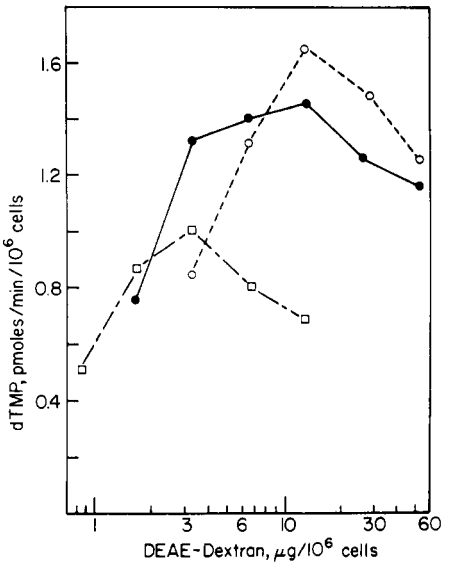

FIGURE 2: Effects of varying DEAE-dextran and cell concentration on incorporation of label from [^{3}H]dTTP into lymphocyte DNA. Incubations (10 min) were carried out under standard conditions, except for varying concentrations of DEAE-dextran (abscissa) and three different concentrations of cells: 0.39 × 10^7/mL (○); 1.56 × 10^7/mL (●); and 6.3 × 10^7/mL (□). With DEAE-dextran omitted, the incorporations were 0.14, 0.17, and 0.14 pmol min^{-1} (10^6 cells)$^{-1}$, respectively.

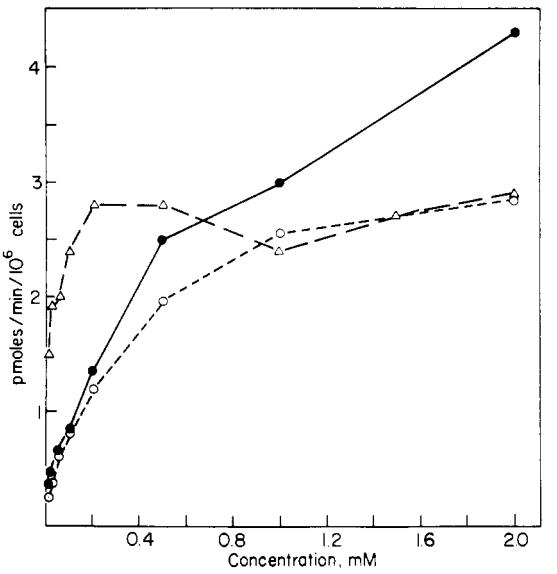

FIGURE 3: The relationship between substrate concentration and incorporation rate, for dTTP, dTMP, and dThd. Incubations (5 min) employed standard conditions, except for varying concentrations of the labeled substrate, which in three different incubations was [^{3}H]dTTP (●), [^{3}H]dTMP (○), and [^{3}H]dThd (△); dTTP was omitted when either [^{3}H]dTMP or [^{3}H]dThd was present.

TABLE II: Distribution of Label from [^{3}H]dTTP after Incubation of Lymphocytes with DEAE-dextran.[a]

Incubation conditions	Incubation time (min)	Distribution of ^{3}H (%) dTTP	dTDP	dTMP	dThd
Complete mixture	0	97	2	1	<0.5
Complete mixture	5	75	18	5	0.8
Complete mixture	10	61	31	7	1
Complete mixture	30	39	21	37	1
Complete mixture	60	29	15	52	2
−ATP	5	11	50	34	2
−phosphoenolpyruvate	5	64	22	5	0.6
−ATP, −phosphoenolpyruvate	5	3	50	36	5

[a] Aliquots of medium from selected incubations in Table I (plus additional incubation times for complete mixture) were analyzed (after removal of cells) by thin-layer chromatography (Experimental Procedure).

TABLE III: Effect of DEAE-dextran on Incorporation of ^{3}H- and ^{32}P-Labeled Deoxynucleotides into DNA.[a]

Labeled substrate	DEAE-dextran	Incorp (pmol/10^6 cells) ^{3}H 3 min	^{3}H 10 min	^{32}P 3 min	^{32}P 10 min
dTMP	−	1.1	17.9	<0.1	<0.1
	+	2.9	16.1	3.7	12.4
dTDP	−	0.1	2.9	<0.1	<0.1
	+	3.3	11.8	3.9	12.0
dTTP	−	0.4	2.1	<0.1	<0.1
	+	3.8	14.1	5.0	15.0
dGTP	−	3.9	24.0	<0.1	<0.1
	+	9.7	37.5	5.3	14.2
dThd	−	3.6	18.6		
	+	4.5	11.3		

[a] Except for the incubation with [^{3}H]dThd, two labels, ^{3}H (100 cpm/pmol) and α-^{32}seP (90 cpm/pmol), were present in the same incubation mixture, in the nucleotide shown. When present, dTMP, dTDP, or dThd was at 0.5 mM and dTTP was not present. Otherwise, standard conditions were used, with incubation for 3 and 10 min and DEAE-dextran present (150 μg/mL) or omitted, as indicated.

at 8 mM, 82% at 4 mM, and 74% with no Mg^{2+} in the incubation mixture.

Even high concentrations of dNTP substrate do not "saturate" the DEAE-dextran system (Figure 3). This may be compared to a broken cell system for lymphocyte DNA synthesis, prepared with a nonionic detergent that lyses plasma membranes but leaves the nucleus intact (Tseng and Goulian, 1975a). Although initial rates for both DEAE-dextran-treated cells and the lysate system are similar, the lysate is saturated at approximately 50 μM, and the apparent "k_s" is 5 μM. In contrast to the triphosphate, the deoxynucleoside saturates the DEAE-dextran system at approximately 0.2 mM, whereas the monophosphate is intermediate in its behavior (Figure 3).

Progressive degradation of substrate occurs during the incubation (Table II), and the rate is very similar in the presence and absence of DEAE-dextran (not shown). The dephosphorylation appears to represent activity of a cell-surface phosphohydrolase, since the degradative activity is not reduced by additional washes of the cells, and it was not found in either the growth medium or the DEAE-dextran mixture after incubation. ATP and phosphoenolpyruvate reduce the dephosphorylation of substrate (Table II); however, since, as shown below, the mono- and diphosphates are approximately equal in activity to the triphosphate, an intracellular function for ATP and phosphoenolpyruvate is the more likely explanation for their stimulatory effect (Table I). The small stimulation by glucose probably has the same basis, i.e., through generation of ATP. A function for ATP other than that of sustaining intra- or extracellular nucleotide phosphorylation, by kinase reactions or protection from phosphohydrolases, could not be distinguished because of the rapid degradation of nucleotides in its absence.

Form of Nucleotide That Enters the Cell. The facile incorporation of label from [α-^{32}P]dTTP into DNA (Table III)

TABLE IV: Deoxynucleotide Incorporation and Concurrent Changes in Concentration and Label Distribution in Lymphocyte DEAE-dextran Incubations Containing Labeled dThd Nucleotides.[a]

Initial Concn[b] (mM)			Incorp[c] (cpm)		Distribution of label in medium (%)[d]					
					^{32}P			^{3}H		
[^{32}P]dTTP	[^{3}H]dTDP	[^{3}H]dTMP	^{32}P	^{3}H	dTTP	dTDP	dTMP	dTTP	dTDP	dTMP
0.5			6040		86	13	0.6			
0.05			2300		80	19	0.6			
	0.5			7290				54	44	0.7
	0.05			2430				80	19	0.6
		0.5		7260				0.7	1.9	95
		0.05		2170				1.3	1.6	91
0.5	0.05		7240	855	82	17	0.7	66	32	0.8
0.05	0.5		689	7580	81	18	0.7	56	42	0.7
0.5		0.05	5850	680	85	14	0.6	0.7	1.5	95
0.05		0.5	1104	5170	88	11	0.6	0.7	1.4	96

[a] Incubation (2 min) employed standard conditions except for the presence of [α-^{32}P]dTTP, [^{3}H]dTDP, or [^{3}H]dTMP, alone and in the combinations and the concentrations indicated (dCTP, dGTP, and dATP were present in all cases but dTTP was present only when stated). [b] Specific activities were the same in all cases, for both ^{32}P and ^{3}H (1200 cpm/pmol); thus, cpm can be compared directly (in the single-label experiments, i.e., lines 1-6) for incorporation of both labels, and for different nucleotides and different initial concentrations. [c] Incorporation into DNA was determined on samples containing 2×10^6 cells. [d] Portions of incubation medium taken at 2 min were assayed by paper chromatography for the distribution of nucleotide label (Experimental Procedure). Both $^{32}P_i$ (determined by nonadsorption to charcoal) and [^{3}H]dThd were less than 1% in all cases.

TABLE V: Comparison of Deoxynucleotide Incorporation and Specific Activity following Incubation of a Mixture Containing both dTTP and dTDP.[a]

Initial Concn (mM)		Concn at 2 min (mM)[b]				Rel Sp Act. at 2 min[c]		Incorp of ^{32}P, 2 min (cpm)[d]
		dTTP		dTDP				
[^{32}P]dTTP	[^{3}H]dTDP	From [^{32}P]dTTP	From [^{3}H]dTDP	From [^{32}P]dTTP	From [^{3}H]dTDP	[^{32}P]dTTP	[^{32}P]dTDP	
0.05	0.5	0.040	0.28	0.009	0.21	0.125	0.041	689
0.5		0.43				1		6040
	0.5				0.22		(1)[e]	(7290)[e]

[a] Calculated from the data of Table IV. [b] Refers to the contributions to total concentration at 2 min separately identified (by ^{32}P or ^{3}H) as derived from the initial [^{32}P]dTTP or [^{3}H]dTDP; obtained by multiplying initial concentrations by the proportion of label in that nucleotide at 2 min. [c] Specific activity at 2 min compared to specific activity of [^{32}P]dTTP at 0 time; for the double-label experiment (line 1) this is obtained by dividing the contribution from that labeled nucleotide (at 2 min) by the total concentration for that nucleotide at 2 min (i.e., [^{32}P]dTTP, 0.04 mM/(0.04 mM + 0.28 mM); [^{32}P]dTDP, 0.009 mM/(0.009 mM + 0.21 mM)); for the single-label experiments (lines 2 and 3), specific activity does not change with incubation. [d] Incorporation of radioactive label into DNA, taken directly from Table IV. [e] Specific activity and incorporation are given for [^{3}H]dTDP for comparison.

indicates that it enters the cells as a deoxynucleotide and is utilized without removal of the phosphate, since only a negligible portion of the α-^{32}P label is converted to $^{32}P_i$, which is diluted by the large excess of nonradioactive P_i in the incubation medium. However, it is not apparent from this whether entry of deoxynucleotide occurs as mono-, di-, or triphosphate, since all three are present even at early incubation times (Table II). Direct comparison by incubation separately with labeled dTMP, dTDP, or dTTP shows that the rates of incorporation of all three into DNA, in the presence of DEAE-dextran, are quite similar (Table III; Table IV, lines 1-6). Analysis of the nucleotides present in the incubation medium indicates that dTMP enters unaltered, since there is very little conversion to di- or triphosphate (Table IV, lines 5, 6, 9, and 10).

The results with labeled tri- and diphosphate substrate are more complex because of large and rapid interconversions between the two (Table IV). However, by using [α-^{32}P]dTTP and [^{3}H]dTDP in the same incubation mixture, and comparing the separate label incorporations and specific activities, it is possible to distinguish between direct entry of the ^{32}P as [^{32}P]dTTP and entry after conversion to [^{32}P]dTDP. With initial concentrations of 0.05 mM [α-^{32}P]dTTP and 0.5 mM [^{3}H]dTDP, by 2 min the specific activity of [^{32}P]dTTP is greatly reduced (Table V), as a result of the combined effects of a reduced amount of ^{32}P label in dTTP (by partial conversion to dTDP), and increased total concentration of dTTP (by formation from [^{3}H]dTDP). The fall in specific activity to 12% compares well with the reduction in ^{32}P incorporation (689 cpm) to 11% of the value for dTTP alone (6040 cpm). (The comparison is made here between the incubations containing the mixture 0.05 mM dTTP + 0.5 mM dTDP, and the 0.5 mM dTTP alone, because of the close correspondence of the concentrations of dTTP for these two incubations at 2 min (0.32 and 0.43 mM, respectively).) By contrast, the specific activity of [^{32}P]dTDP, formed from [^{32}P]dTTP in the same mixture, is reduced much further, to 4% (whereas the incorporation of [^{3}H]dTDP alone (7250 cpm) is similar to that of [^{32}P]dTTP alone). Thus, the results are best accounted for by direct entry of dTTP into the cell, without prior conversion to di- (or mono-) phosphate.

The preceding analysis compares incorporation, which reflects rate from 0 time, with substrate composition at 2 min; however, experiments not shown indicate that the interconversions between di- and triphosphate occur very rapidly (i.e., predominantly by 30 s). The data from 5 min allow interpretations similar to those at 2 min and are not illustrated. Because

TABLE VI: Effect of Omitting 1 dNTP.[a]

Labeled substrate	Substrate mixture	Incorp (pmol/10^6 cells) 3-min incubation −Hydroxy urea	3-min incubation +Hydroxy urea	10-min incubation −Hydroxy urea	10-min incubation +Hydroxy urea
[^{3}H]dTTP	Complete	3.07	3.06	9.66	9.78
[^{3}H]dTTP	−dCTP	2.29	1.98	10.90	5.69
[^{3}H]dTTP	−dATP	2.48	1.88	9.38	2.42
[^{3}H]dTTP	−dGTP	1.38	0.47	6.01	0.85
[^{3}H]dCTP	Complete	4.48	4.56	11.91	12.80
[^{3}H]dCTP	−dTTP	3.93	4.31	11.49	12.25
[^{3}H]dCTP	−dATP	3.69	3.03	11.66	3.61
[^{3}H]dCTP	−dGTP	2.74	1.03	5.07	1.48

[a] Incubations of lymphocytes were carried out with DEAE-dextran under standard conditions with the ^{3}H label in either dTTP or dCTP, and omission of single (unlabeled) dNTPs, as indicated. Hydroxyurea when present was at 2 mM.

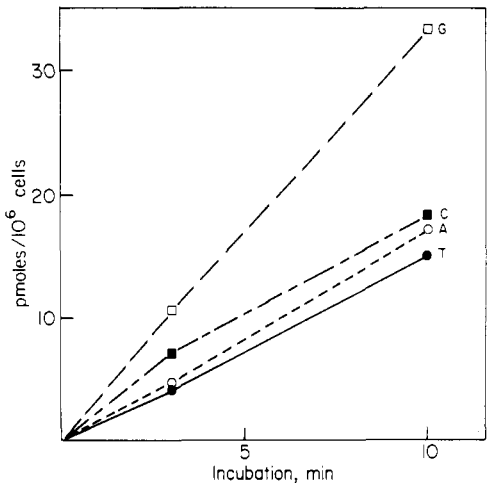

FIGURE 4: Incorporation of different ^{3}H-labeled dNTPs. Standard conditions were used, except that, in separate incubations, the ^{3}H label was in either [^{3}H]dTTP (●), [^{3}H]dATP (○), [^{3}H]dCTP (■), or [^{3}H]-dGTP (□).

of the large and rapid conversion of dTDP to dTTP the data of Tables IV and V cannot be used to test for direct entry of dTDP. However, since both deoxynucleoside mono- and triphosphates appear to enter with equal facility (Tables III, IV, and V), we presume that the same applies to the diphosphate as well.

There is a pronounced incorporation of radioactive label from [^{3}H]dTMP and [^{3}H]dGTP in the absence of DEAE-dextran (Table III); however, this is not seen with the corresponding α-^{32}P-labeled deoxynucleotide and we infer that this occurs via the deoxynucleosides (dThd, dGuo) in each case. The apparent increase in rate from 3 to 10 min is consistent with this, reflecting increased availability of the deoxynucleoside during incubation. The nucleoside is utilized more efficiently at low concentrations, as compared to the nucleotide (Figure 3) but, at the usual concentration for substrate in these incubations (0.5 mM), the rate of incorporation of labeled dThd is similar to the rates observed with the deoxynucleotides (Figure 3 and Table III). The small residual incorporation of label from [^{3}H]dTDP and [^{3}H]dTTP, in the absence of DEAE-dextran, may also be ascribed to the utilization of nucleoside, since it is not detected for either [α-^{32}P]dTDP or [α-^{32}P]dTTP (Table III).

Cellular Deoxynucleotides. From experiments not illustrated here in which the fate of in vivo labeled dThd nucleotide pools was followed (Experimental Procedure), it is evident that cellular deoxynucleotide pools leak into the medium. For example, 75% of the prelabeled dThd nucleotides was found in the medium after 3 min of incubation under standard conditions with DEAE-dextran, and 85% after 10 min. Leakage of cellular deoxynucleotides also occurs without DEAE-dextran albeit less than in the presence of DEAE-dextran.

The effect of omitting single dNTPs from the incubation mixture varies with the particular dNTP. When dGTP is omitted, the rates of incorporation of either [^{3}H]dTTP or [^{3}H]dCTP drop to approximately one-half, whereas omission of dTTP causes no effect, as measured by incorporation of label from [^{3}H]dCTP (Table VI). The requirement for either dCTP or dATP appears intermediate; omission of either results in a small reduction in synthesis at 3 min but no detectable effect with longer incubations. The presence of hydroxyurea magnifies the effect of omitting a dNTP, apparently by blocking de novo synthesis of dNTPs through its inhibitory effect on ribonucleotide reductase (Elford, 1968). These results suggest that the DEAE-dextran-treated cells contain residual intracellular deoxynucleotide pools that are at least partially distinct from the medium. In the presence of hydroxyurea, the relative sizes of these pools appear to be dTTP > dCTP > dATP > dGTP (Table VI), which is the same order determined directly for mouse embryo cells in the presence of the same inhibitor (Skoog and Nordenskjöld, 1971).

In spite of the aforementioned evidence for residual precursor pools of different sizes, there was little difference in incorporation between [^{3}H]dTTP, [^{3}H]dATP, and [^{3}H]dCTP (Figure 4). The higher incorporation rate of label from [^{3}H]dGTP (Figure 4) is thought to have another explanation (see above), since [α-^{32}P]dGTP was incorporated at essentially the same rate as for dThd nucleotides (Table III). Experiments in parallel to those shown in Figure 4 and Table III, but with the inclusion of hydroxyurea, gave very similar results.

Nature of the DNA Synthesized. Following incubation of cells with [^{3}H]dTTP and DEAE-dextran, cytoplasmic membranes were lysed with nonionic detergent, and nuclei were isolated and washed (Experimental Procedure). All of the radioactive label incorporated into DNA in the DEAE-dextran-treated cells could be accounted for by nuclear DNA synthesis.

DNA was prepared from DEAE-dextran-treated cells incubated with BrdUTP in place of dTTP, and [^{3}H]dCTP in place of [^{3}H]dTTP. The product was sheared and then centrifuged to equilibrium in CsCl (Figure 5). The hybrid density of the product supports a mechanism of semiconservative replication. This result also indicates that the cellular pool of dTTP does ultimately equilibrate with the incubation medium. However, consistent with the other evidence in support of partially separate cellular deoxynucleotide pools (see above), densities intermediate between hybrid and light resulted when the experiment did not include preincubation with BrdUTP (see Experimental Procedure).

DNA from cells labeled for various times with [^{3}H]dTTP in the presence of DEAE-dextran was centrifuged in alkaline sucrose gradients, and the results are compared with a similar analysis of cells labeled in culture with [^{3}H]dThd (Figure 6). In both the DEAE-dextran-treated cells and in the growing culture, a large part of the earliest labeled product is in the form of short fragments. With longer centrifugation times, the short fragments have a distribution around 4 S (data not shown). In both in vitro and in vivo, with longer labeling times

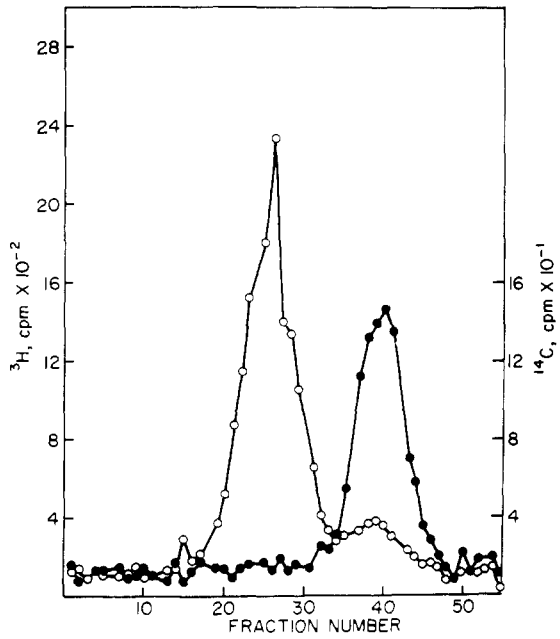

FIGURE 5: Equilibrium density gradient centrifugation of BrdUMP-containing DNA synthesized in DEAE-dextran-treated lymphocytes. Cells prelabeled with [^{14}C]dThd were incubated with [^{3}H]dCTP and BrdUTP in place of dTTP. Details of the procedure, including a preincubation with BrdUTP, are given in Experimental Procedure. DNA was extracted and centrifuged to equilibrium in CsCl. Density increases from right to left. Densities at fractions 25 and 40 were 1.750 and 1.700 g/cm^3, respectively: (●) ^{14}C; (○) ^{3}H.

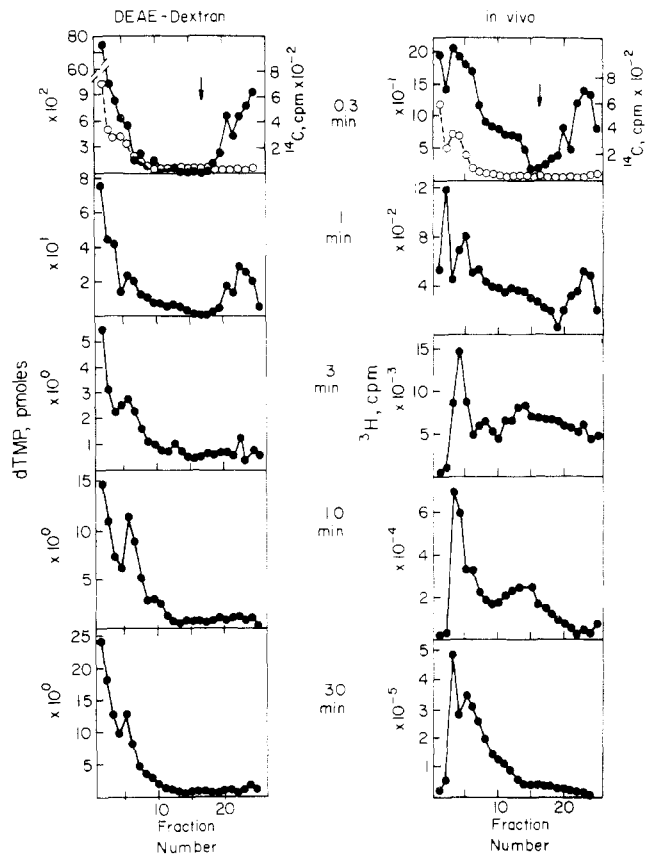

FIGURE 6: Alkaline sucrose gradient centrifugation of DNA product pulse labeled in DEAE-dextran-treated cells and in growing culture. DNA was labeled with [^{3}H]dTTP in DEAE-dextran-treated lymphocytes for 20 s and 1, 3, 10, and 30 min, and for similar intervals in vivo with [^{3}H]dThd (Experimental Procedure). The DNA from both types of preparation was analyzed by velocity centrifugation in alkaline sucrose gradients. ^{14}C from bulk DNA prelabel is shown only for the 20-s samples; position of an fd DNA (20 S) marker is indicated by the arrow (fraction 16). Direction of sedimentation is from right to left: (●) ^{3}H; (○) ^{14}C.

the amount of low-molecular-weight DNA remains relatively constant as high-molecular-weight bulk DNA continues to accumulate at the bottom of the gradients. These results are consistent with other experiments that have shown that the DNA is synthesized in short fragments, which serve as precursor for high-molecular-weight DNA (Schandl and Taylor, 1969; Nuzzo et al., 1970; Fox et al., 1973; Huberman and Horwitz, 1973; Tseng and Goulian, 1975a). A pulse-chase type of experiment in which label incorporation ceases with the addition of excess unlabeled precursor could not be carried out in either the cultures or the DEAE-dextran incubations, presumably because of retained precursor pools.

Thus, along with restriction of synthesis to S phase, the evidence for semiconservative synthesis, and for a discontinuous mechanism, indicates that DNA synthesis in DEAE-dextran cells resembles in vivo DNA synthesis.

Effect of DEAE-dextran on the Cells. Simple addition of DEAE-dextran to cells in culture medium at 0.1 to 10 mg/mL, under normal conditions of growth, results in no detectable effect on appearance by phase microscopy, or viability to trypan blue dye exclusion, and at 0.1 mg/mL did not alter growth or doubling times. However, incubation at 37 °C with DEAE-dextran in the standard mixture for these experiments did result in loss of viability (trypan blue), by $\frac{1}{3}$ in 1 min, $\frac{1}{2}$ in 10 min, and $\frac{2}{3}$ in 30 min. When the incubated cells were washed and returned to the growth medium, there was neither growth nor further change in viability in the subsequent 20 h.

It is not known why, in the DEAE-dextran system, incorporation of deoxynucleotide into DNA slows and stops with only a small extent of replication completed (see Discussion). Experiments that tested for the effect of cell density or exhaustion of nucleotide substrates have made it clear that cessation of synthesis is not related to either.

The ability of DEAE-dextran to enhance uptake of deoxynucleotides is not restricted to cultured lymphocytes. Two other cell lines, L cells and HeLa cells, were tested and both gave results similar to the lymphocyte, both in kinetics of incorporation and optimum DEAE-dextran concentration.

Discussion

The experiments described here show that DEAE-dextran facilitates uptake of deoxynucleotides into cultured animal cells, allowing incorporation into DNA. In the absence of DEAE-dextran there was no incorporation of α-^{32}P-labeled deoxynucleotides above the level required for detection in these experiments (0.1 pmol/10^6 cells). There have been reports of the uptake by untreated animal cells of certain nucleotide analogues (e.g., arabinosylcytosine monophosphate), albeit at very low levels (Plunkett et al., 1974; LePage and Naik, 1975).

Several experiments were carried out to determine whether deoxynucleotides in the DEAE-dextran-treated cell are replaced by deoxynucleotides from the incubation medium. The fact that most prelabeled cellular deoxynucleotide is lost from the cells during incubation, and that rates of incorporation for all four dNTPs are similar in spite of different sizes of residual cellular pools for each, suggest (but do not prove) that there is relatively little dilution of deoxynucleotide label by residual cellular deoxynucleotide pools.

In apparent conflict with this are two results that indicate incomplete replacement of cellular deoxynucleotide with deoxynucleotide from the incubation medium: inability to dilute

a short pulse label with excess unlabeled deoxynucleotide, and inability to replace cellular dTTP pools with BrdUTP (unless a preincubation with BrdUTP is included). These results can be reconciled if it is assumed that at the start of incubation cellular deoxynucleotide pools are at least partially distinct from the medium, but that equilibration takes place during the first few minutes of incubation. If this is the case, the apparent linearity of the initial period of incorporation may result from the opposing effects of progressively increasing specific activity of cellular precursor pools and diminishing rate of synthesis. Thus, the initial rate of incorporation may underestimate the rate of DNA synthesis due to the dilution of labeled precursor by preexisting pools.

DEAE-dextran appears to enhance uptake of nucleic acid into cells by interacting with both the nucleic acid and the cell surface (Pagano, 1970). As with nucleic acids, nucleotides may form a complex with DEAE-dextran on the basis of their charge difference, thereby facilitating entry into the cell. Pinocytosis has been proposed as the mechanism for entry of nucleic acids (Pagano, 1970); however, it is not known whether DEAE-dextran actually enters the cell in association with the negatively charged molecule.

The process of DNA synthesis in the DEAE-dextran-treated cell resembles its counterpart in intact cells by the three criteria tested here, i.e., synthesis in S phase cells, occurring by semiconservative and discontinuous mechanisms. The duration of DNA synthesis is short, for reasons that are not understood, although the initial rate is high and may even approach the in vivo rate. The total extent of synthesis achieved with broken cell systems (Hershey et al., 1973; Tseng and Goulian, 1975a) and with a previously reported permeabilized cell system (Seki et al., 1975) is also limited to values similar to the level found with the DEAE-dextran permeabilized cells. It is possible that, similar to prokaryote in vitro systems (Gefter, 1975), the observed synthesis represents completion of replication in units in which synthesis was already in progress, but that new initiations do not take place.

The defined nuclear structure of the animal cell, which can be isolated relatively intact, may simplify some studies on eukaryote DNA synthesis in vitro (Friedman and Mueller, 1968; Winnaker et al., 1971; Burgoyne, 1972; Kidwell, 1972; Lynch et al., 1972; Probst et al., 1972; Hershey et al., 1973; Lazarus, 1973; Hallick and Namba, 1974; Hunter and Francke, 1974; Tseng and Goulian, 1975a). However, the DEAE-dextran system, along with the other recently described permeabilized cell systems (Seki et al., 1975; Berger and Johnson, 1976), can complement broken cell systems for studies in which nucleotide incorporation into DNA is required in the "intact" cells. One way in which the DEAE-dextran system has been applied for this purpose is the identification of RNA–DNA junctions in animal cells by a form of nearest-neighbor experiment that requires incorporation of [α-^{32}P]deoxynucleotides into DNA (Tseng and Goulian, 1975b). DEAE-dextran may also facilitate uptake of ribonucleotides and proteins, but neither has yet been tested in this system.

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