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Biochemistry

Multienzyme complex for metabolic channeling in mammalian DNA replication

(replitase/ribonucleoside diphosphate reductase/permeabilized cells/cell cycle)

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ABSTRACT **In the DNA-synthesizing phase (S phase) of CHEF/18 Chinese hamster embryo fibroblast cells, six enzymes associated with DNA metabolism, including DNA polymerase (deoxynucleoside triphosphate:DNA deoxynucleotidyl-transferase, EC 2.7.7.7), were largely localized in the nuclear region (karyoplasts). By contrast, in quiescent and G_1 phase cells these enzymatic activities were mainly absent from the nucleus and were recovered in the cytoplasmic portion (cytoplasts). These nuclear (but not cytoplasmic) enzymatic activities cosedimented rapidly on sucrose density gradients. Further, the rapidly sedimenting enzyme activities were unique to cells in S phase. An organized supramolecular structure that allows channeling of metabolites into DNA was demonstrated by kinetics of nucleotide incorporation. "Permeabilized" cells selectively channeled incorporation of ribonucleoside diphosphates into DNA in preference to deoxyribonucleoside triphosphates. Deoxyribonucleoside triphosphate incorporation occurred when ribonucleoside-diphosphate reductase (2′-deoxyribonucleoside-diphosphate: oxidized-thioredoxin 2′-oxidoreductase, EC 1.17.4.1) activity was abolished by hydroxyurea. Our interpretation is that during DNA replication, the nucleus contains a complex of DNA precursor-synthesizing enzymes juxtaposed with the "replication apparatus" comprising DNA polymerase, other enzymes, and structural proteins. Functional integrity of this structure is impaired when one of its essential components is inactivated. We propose the name "replitase" for this multienzyme complex for DNA replication and suggest that it incorporates precursors rapidly and efficiently. Possibly its assembly signals the initiation of the S phase of the cell cycle.**

Replication points of DNA in a variety of mammalian cells are situated on the nuclear membrane (1–4), and the replication starts at the nuclear membrane (5). This association takes place with the aid of newly synthesized protein(s) 2–4 hr before commencement of the S phase (6). Membrane–DNA aggregates have been isolated from bacteria (7), from developing sea urchin nuclei (8), and from regenerating rat liver mitochondria (9) only in cells undergoing DNA synthesis. These aggregates could synthesize DNA from deoxyribonucleoside triphosphates (dNTPs) *in vitro* (8, 10, 11). The enzymes of DNA replication thus might be nuclear—bound to nuclear membrane and to DNA at the time of DNA replication.

DNA polymerase-α has been shown to be in the nucleus by using the technique of fractionating whole cells into karyoplasts (mainly nuclei) and cytoplasts (12), but other reports have given rather conflicting views. During DNA replication, the enzymes have been reported to be attached to nonnuclear membrane of rat liver cells (13). Ribonucleotide reductase (2′-deoxyribonucleoside-diphosphate: oxidized-thioredoxin 2′-oxidoreductase, EC 1.17.4.1), thymidylate synthase (5,10-Methylene-tetrahydrofolate: dUMP *C*-methyltransferase, EC 2.1.1.45), thymidine kinase (ATP: thymidine 5′-phosphotransferase, EC 2.7.1.21), and DNA polymerase (deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.7) also have been found in aggregates (8.5–12 nm) associated with postmicrosomal membrane fragments of unknown origin in Novikoff tumor cells (14).

In eukaryotic cells, DNA replication occurs only during certain periods and at a limited number of definitive intracellular sites. Rapid rates of DNA replication are maintained (15) despite low precursor pools (16), compared to high concentrations of deoxyribonucleoside triphosphates (dNTPs) required to saturate the replication *in situ* (17). These and other observations relating to the kinetics of incorporation of labeled precursors into DNA and into cellular pools (18, 19) have indicated the existence of concentration gradients of deoxyribonucleotides, with the highest concentrations near the replication forks.

Demonstration of multienzyme complexes in various macromolecular biosynthetic processes (20–22) has replaced the primitive notion that the cell is a mere bag of uniformly dispersed soluble enzymes catalyzing freely diffusing metabolites (for review, see refs. 23 and 24). Several lines of evidence from both prokaryotic and eukaryotic systems indicated that DNA precursors are compartmentalized in replicating cells (18, 19, 25). Mathews and coworkers (26, 27) and Greenberg and coworkers (28, 29) have demonstrated that, in bacteriophage T4-infected cells, compartmentation and metabolite channeling for DNA replication are facilitated by the existence of protein–protein interactions. A similar functional compartmentation in eukaryotic cells as well might occur.

Therefore, we explored the existence and functional significance of multienzyme complexes in DNA replication of eukaryotic cells by (*i*) analyzing the intracellular distribution of some key enzymes at different stages of the cell cycle, (*ii*) determining the activity of those enzymes in the rapidly sedimenting material of nuclear lysates, and (*iii*) examining the kinetics of ribonucleoside diphosphate (rNDP) and dNTP incorporation into cells made permeable by treatment with lysophosphatidylcholine ("permeabilized cells").

METHODS AND MATERIALS

Cells and Culture Conditions. The Chinese hamster embryo fibroblast cell line, CHEF/18 (obtained from Ruth Sager), was maintained in a humidified, 10% CO_2 incubator at 37°C in Dulbecco's modified Eagle's medium (Flow Laboratories,

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Abbreviations: dNTPs, deoxyribonucleoside triphosphates; rNDPs, ribonucleoside diphosphates; NDP kinase, nucleosidediphosphate kinase (ATP: nucleosidediphosphate phosphotransferase, EC 2.7.4.6); dCMP kinase, deoxycytidine monophosphate kinase.

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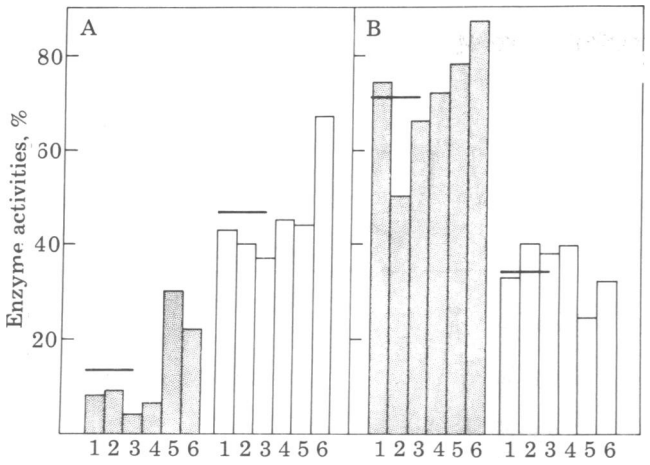

FIG. 1. Percentage of enzyme activities present in karyoplasts (*A*) and cytoplasts (*B*) of quiescent and growing cells in logarithmic phase. These percentages are calculated in relation to total activities recovered in the corresponding extracts of whole cells (data not shown). The activities recovered in the karyoplasts and cytoplasts were corrected for cross-contamination. Horizontal thick lines over each set of bars represent the average percentage of all the enzyme activities present in that particular group. Shaded bars, quiescent cells; open bars, growing cells. 1, DNA polymerase; 2, thymidine kinase; 3, dCMP kinase; 4, NDP kinase; 5, thymidylate synthetase; and 6, dihydrofolate reductase.

McLean, VA) with 10% (vol/vol) fetal calf serum (Microbiological Associates, Bethesda, MD) plus 100 μg of streptomycin per ml and 100 units of penicillin. All cultures were determined to be mycoplasma-free by the method of Schneider *et al.* (30).

At least 90% of the cells in a confluent culture were not in S phase, as determined by flow microfluorimetry (31). About 50% were in S phase 36–48 hr after replating of the cells at lower density. Confluent cultures were defined as quiescent and as growing 36–48 hr after replating. Cells that were arrested by placing them in isoleucine-deficient medium for about 36 hr progressed synchronously into S phase after they were placed in fresh medium containing isoleucine (32).

Preparation of Karyoplast and Cytoplast Lysates. Cells were fractionated into nuclear (karyoplasts) and cytoplasmic (cytoplasts) components essentially as described by Lucas and Kates (33). Karyoplasts collected at the bottom of the tube as a pellet were suspended in fresh medium, and cytoplasts remaining attached to the plates were scraped into medium using a rubber policeman. They both were pelleted and resuspended in sucrose buffer (0.25 M sucrose/50 mM Tris·HCl, pH 7.6/25 mM KCl/5 mM Mg acetate/1 mM dithiothreitol) at a density of 10^8 particles per ml. They were disrupted with a Branson Sonifier (model W-185 E) at the lowest setting for 15–20 sec and clarified by centrifugation for 5 min in a Sorvall RC-5B superspeed centrifuge at 2000 × *g* and 4°C. Cytoplasmic contamination (12%) of the karyoplasts was estimated by assaying for NADH dehydrogenase activity (34). Nuclear contamination (5%) of cytoplasts was estimated by staining with Giemsa and by microscopic counting.

Enzyme Assays. DNA polymerase: The reaction mixture contained 60 μl of lysate combined with 90 μl of assay solution to give final concentrations of 35 mM Hepes (pH 7.4), 50 mM sucrose, 80 mM KCl, 4 mM $MgCl_2$, 10 mM phosphoenolpyruvate, 30 μg of calf thymus DNA ["activated" by pancreatic DNase (35)], 1.25 mM ATP, and 0.25 mM each of [^{3}H]dTTP (180 cpm/pmol; Amersham), dATP, dCTP, and dGTP. After 30 min at 37°C, acid-precipitable radioactive material was determined (36).

Thymidine kinase (ATP: thymidine 5′-phosphotransferase, EC 2.7.1.21): Lysate (75 μl) in a total volume of 200 μl containing 0.01 M Tris (pH 7.8), 5 mM $MgCl_2$, 5 mM ATP, and 57 μM [^{3}H]thymidine (880 cpm/mol) (New England Nuclear) was incubated for 60 min at 37°C. The [^{3}H]thymidine nucleotide formation was determined (37).

NDP kinase (38), dihydrofolate reductase (tetrahydrofolate dehydrogenase, EC 1.5.1.3) (39), thymidylate synthase (5,10-Methylene-tetrahydrofolate:dUMP C-methyltransferase, EC 2.1.1.45) (40), dCMP kinase (41), and protein (42) were assayed as described.

Sedimentation Analysis of Enzymes. Lysates were prepared, clarified, and dialyzed for 3 hr against 1000 vol of dialysis buffer (50 mM Tris·HCl, pH 7.8/4 mM $MgCl_2$/7 mM 2-mercaptoethanol). Four-tenths ml of dialyzed lysate was layered over a 5-ml linear gradient of 5–20% (wt/vol) sucrose in dialysis buffer, which in turn was layered over a 66% sucrose pad (0.4 ml) and centrifuged in a Beckman SW 50.1 rotor at 4°C for 8 hr at 35,000 rpm. The gradient was resolved into 0.5-ml fractions that were assayed for enzyme activities.

DNA Synthesis in Permeabilized Cells. Cells in suspension were permeabilized by treating them with 250 μg of L-α-lysophosphatidylcholine per ml (43). The dNTP-incorporation mixture contained in 0.3-ml 35 mM Hepes (pH 7.4); 50 mM sucrose; 80 mM KCl; 4 mM $MgCl_2$; 7.5 mM potassium phosphate (pH 7.4); 0.75 mM $CaCl_2$; 10 mM phospho*enol*pyruvate; 1.25 mM ATP; 0.12 mM each of CTP, UTP, and GTP; 0.25 mM each of [^{3}H]dTTP (88 cpm/pmol), dATP, dCTP and dGTP, and 2 × 10^6 permeabilized cells. The rNDP-incorporation mixture was the same except that 10 mM formaldehyde, 2 mM *dl*-L-tetrahydrofolate (Sigma), 8 mM dithiothreitol, and 0.25 mM [^{3}H]CDP (55 cpm/pmol; Amersham) were added and UDP, GDP, and ADP were used in place of dNTPs. After incubation for 10 min at 37°C, acid-precipitable, alkali-resistant material was prepared and measured for radioactivity (36).

RESULTS

Intracellular Localization of the Enzymes Associated with DNA Metabolism. The locations of several DNA precursor-synthesizing enzymes in quiescent and growing CHEF/18 cells are shown in Fig. 1. On the average only about 13% of these enzyme activities was present in the nuclear component (karyoplasts) of quiescent cells, in contrast to about 50% in the nuclear component of growing cultures. These percentages correspond with the proportion of the cells that were making DNA in the respective cultures. In accordance with these relocations in karyoplasts, opposite changes were reflected in the corresponding cytoplasts. These results suggest the appearance of enzyme molecules in the nucleus as a preparation for S phase.

This aspect was further explored by examining the distributions of representative enzymes as the cells progressed from G_0 through G_1 phase to S phase. Cells arrested in G_0 by isoleucine deprivation progressed into S phase 6–12 hr after they were placed in fresh complete medium (see Fig. 4). The specific activities for several enzymes increased in the karyoplasts of these cells 6–8 hr after release from G_0 arrest (Fig. 2), which corresponded to the time when nuclear DNA started to replicate.

De novo enzyme synthesis in the nucleus must be considered as an alternative to migration from the cytoplasm. The total cellular activity of NDP kinase remained unchanged as cells progressed from G_0 to S, but its activity increased in karyoplasts with a concomitant decrease within cytoplasts (Fig. 2 *Top*), indicating migration of this enzyme into the nucleus.

Preferential cytoplasmic location of these enzymes in nongrowing cells could occur also if the nuclei became much smaller during quiescence. However, distribution of total

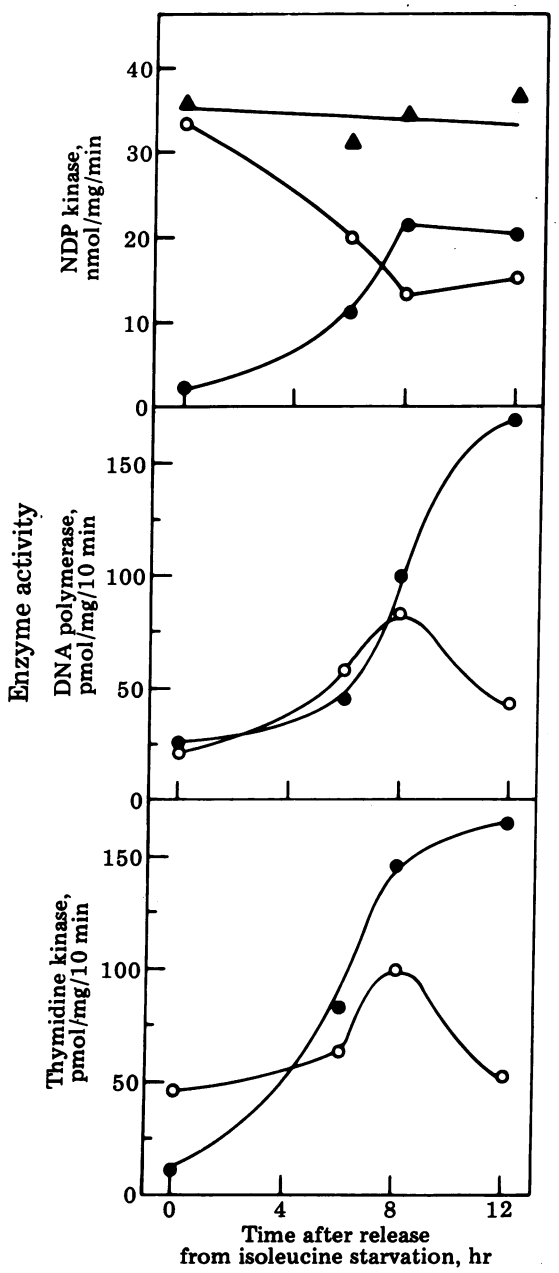

FIG. 2. Changes in specific activity of the enzymes in karyoplasts, cytoplasts, and whole cells progressing from G_0 to S. Cells were arrested in G_0 by using isoleucine-deficient medium. At various intervals after transfer to fresh complete medium, karyoplasts and cytoplasts were prepared. Lysates of these particles were prepared and enzyme activities were measured. ○, Activity in cytoplasts; ●, activity in karyoplasts; ▲, activity in whole cells.

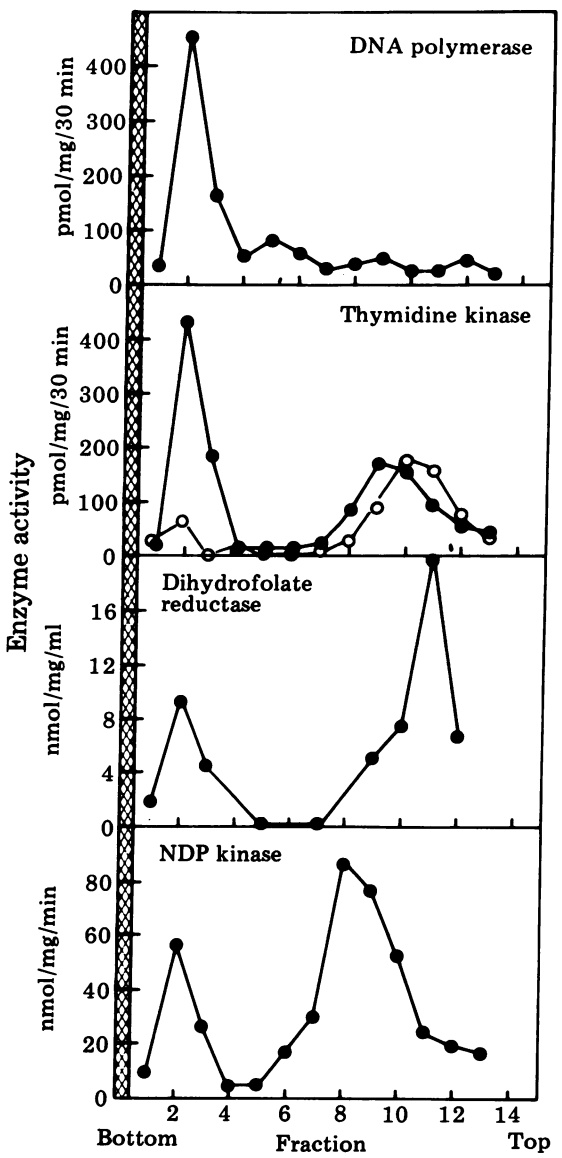

FIG. 3. Sucrose density gradient analysis of enzyme activities in nuclear lysates. Cells were arrested in G_0 by using isoleucine-deficient medium for 12 hr (●, S phase) and 6 hr (○, pre-S phase). After replacement in fresh medium containing isoleucine, karyoplasts were prepared and sucrose density gradient analysis of their lysates was carried out. Sedimentation of enzymes is from right to left.

protein between karyoplasts and cytoplasts of growing and quiescent cells did not reveal any significant difference (data not shown).

These results are consistent with the hypothesis that the enzymes of DNA metabolism, initially located in the cytoplasm of the quiescent cells (and synthesized during G_1), are translocated into nuclei of cells that are about to replicate DNA.

A Multienzyme Complex in S Phase Cells. An unlocalized aggregate of enzymes, including DNA polymerase-α, ribonucleotide reductase, thymidylate synthase, and thymidine kinase, has been reported by Baril *et al.* (14) in Novikoff tumor cells. In order to ascertain the localization and physiological significance of such enzyme complexes, we prepared lysates of karyoplasts of S phase cells. Sedimentation profiles of the activities of four enzymes were performed on sucrose density gradients. A major fraction of these enzymes (but not adenylate deaminase or hypoxanthine phosphoribosyltransferase) cosedimented rapidly (Fig. 3). In contrast, these activities sedimented slowly as expected for free enzymes in the lysates prepared from cytoplasts (data not shown).

The rapidly sedimenting pattern of these enzyme activities was unique to the cells making DNA. Lysates of cells in S phase, but not of cells in G_1 phase, contained a major fraction of thymidine kinase that rapidly sedimented on sucrose gradients (Fig. 3).

Metabolite Channeling in Permeabilized Cells. The primary physiological role of aggregated multienzyme systems lies in their ability to compartmentalize metabolites even in the absence of physical barriers (23, 24). Furthermore, distal or initial substrates of a macromolecular biosynthesis sequence could thereby be utilized more readily than could proximal or ultimate substrates (27). To test channeling, we used permea-

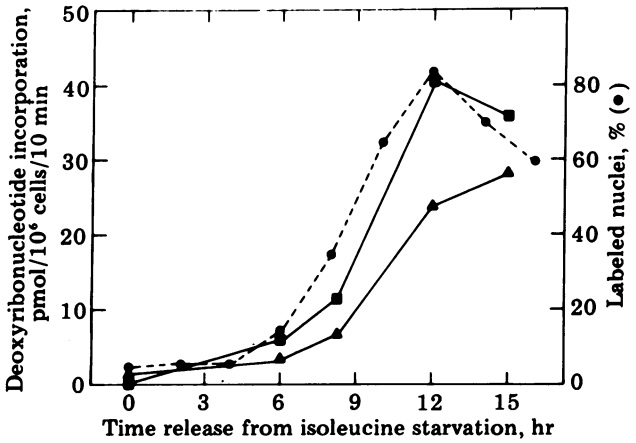

FIG. 4. DNA synthesis in synchronized cells. Cells were arrested by using isoleucine-deficient medium. They were replaced in fresh complete medium. At intervals after release, some plates were trypsinized and the cells were permeabilized in suspension. Permeabilized cells were used to measure DNA synthesis, in the presence of dNTPs (▲) or rNDPs (■). Other cells were exposed to 2 μCi (1 Ci = 3.7 × 10 becquerels) [^{3}H]thymidine per ml for 30 min at 37°C, fixed, and processed for autoradiography, and the percentage of labeled nuclei was determined (●).

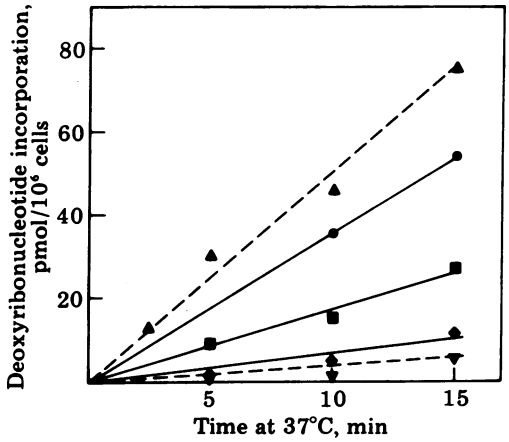

FIG. 5. Incorporation of dNTP and rNDP into DNA of permeabilized cells. Cells were arrested in G_0 by using isoleucine-deficient medium. Twelve hours after release from the isoleucine block, cells were trypsinized and permeabilized. Permeabilized cells were incubated with rNDPs and dNTPs in various incubation mixtures: ▲, rNDPs incubated with the rNDP incorporation mixture; ▼, rNDPs incubated with the rNDP incorporation mixture and hydroxyurea (2 mM); ●, dNTPs incubated with the dNTP incorporation mixture and hydroxyurea (2 mM); ◆, dNTPs incubated with the rNDP incorporation mixture; ■, dNTPs incubated with the rNDP incorporation mixture and hydroxyurea. Acid-precipitable, alkali-resistant material was prepared and radioactivity was measured.

bilized animal cells which, when supplied with deoxyribonucleoside diphosphates or dNTPs as the sole substrates, synthesized DNA at rates comparable to those of intact cells (44). Under these conditions, substitution of rNDPs for dNTPs decreased DNA synthesis to 24% (44). However, when the incubation mixture was modified by provision of dithiothreitol, which is required for activation of rNDP reductase (45, 46), and of formaldehyde and *dl*-L-tetrahydrofolate, which generate methylenetetrahydrofolate for thymidylate synthetase activity, we observed that rNDPs were incorporated into DNA 20% to 25% *more* efficiently than were dNTPs in the original incubation mixture (Fig. 4).

The acid-precipitable material synthesized from rNDPs was indeed DNA, as ascertained by its resistance to alkali. Its synthesis was absolutely dependent on the stage of the cell cycle, in accordance with the incorporation of dNTPs (Fig. 4). Furthermore, reduction of rNDPs to dNDPs prior to their participation in DNA synthesis was revealed by a dependence on the active state of rNDP reductase created by dithiothreitol. In addition hydroxyurea, which is known to inhibit rNDP reductase (47), prevented incorporation of rNDPs but not dNTPs (Fig. 5).

These observations demonstrate that rNDPs permit DNA replication in permeabilized cells under appropriate conditions and do so more efficiently than do dNTPs. This finding is compatible with the rNDPs being accessible to replication forks preferentially through the multienzyme complex.

This metabolic channeling can be disrupted to allow rapid incorporation of dNTPs. This was observed in the original incubation mixture, where rNDP reductase was not activated. Provision of dithiothreitol decreased dNTP incorporation to about 20% (Fig. 5). This situation was partially reversed by adding hydroxyurea to this incubation mixture. The trivial explanation that the inhibitory effect of dithiothreitol on dNTP incorporation is simply because of its influence on DNA polymerase activity can be eliminated by the observation that DNA polymerase activity in crude extracts was decreased only by about 5–10% in the presence of 9 mM dithiothreitol. In contrast, in the permeabilized cells dNTP incorporation into DNA finally decreased by about 90% (Fig. 6).

DISCUSSION

Six enzymes of DNA synthesis were in different locations depending on the stage of cell growth. In growing cells, these enzymes were largely localized in karyoplasts. Furthermore, their specific activities increased in karyoplasts as the cells progressed into S phase, suggesting that the enzymes migrate into the nuclear region when DNA replication is taking place. In contrast, these enzymatic activities were mainly absent from the nucleus and were recovered in the cytoplasmic component (cytoplasts) in quiescent cells.

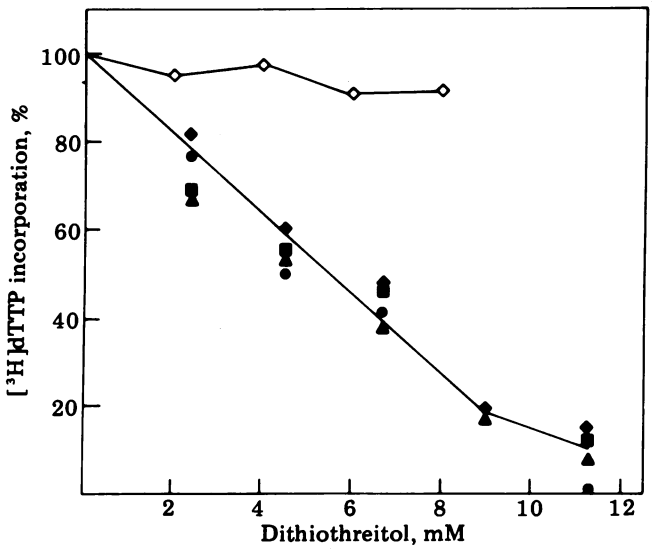

FIG. 6. Influence of dithiothreitol on DNA polymerase activity in crude extracts and permeabilized cells. Cells were arrested in G_0 by using isoleucine-deficient medium. Twelve hours after release from the isoleucine block, cells were trypsinized. Some cells were permeabilized, and others were sonicated to prepare crude extracts. Under increasing concentration of dithiothreitol, permeabilized cells were tested for dNTP incorporation into DNA by incubating for various intervals (●, 4 min; ■, 8 min; ▲, 12 min; and ◆, 16 min). Crude extracts were similarly assayed for DNA polymerase activity (◇).

Mere localization of the DNA precursor-synthesizing enzyme in the nucleus might not provide a very effective means of compartmentalization of dNTPs, owing to the presumably free permeability of the nuclear membrane to small molecules (48), in contrast to demonstrated compartmentalization of dNTPs in mitochondria (49). However, if these enzymes, including DNA polymerase, were assembled into multienzyme complexes, efficient compartmentalization of precursors could be accomplished. Our observations that rNDPs can be incorporated into DNA more efficiently than dNTPs and that dNTPs are actually prevented from incorporation under the conditions suitable for rNDPs suggest effective metabolite channeling inherent in these complexes, as described for prokaryotic systems (27).

Our interpretation of these results is that the complex in a physiologically active state, when rNDP reductase is activated, channels rNDP metabolites directly to the site of their incorporation; the channel is poorly accessible to the proximal dNTP precursors. The functional integrity that is required to channel rNDPs can be absolished by altering the complex, as occurs in extracts, or by inactivating one of its essential components, as accomplished with hydroxyurea or in the absence of dithiothreitol. This metabolically disintegrated complex allows dNTPs to participate directly in DNA replication. In eukaryotic cells the interaction between DNA polymerase and DNA precursor-synthesizing enzymes seems to be more stable than in prokaryotic systems (26), as revealed by cosedimentation. In addition, in our *in vitro* assay system the DNA polymerase activity contained in the complex did not require exogenous "activated" DNA template, indicating that it contains template and newly replicating DNA (unpublished data).

We propose the name "replitase" for this complex that contains the enzymes that make DNA from rNDPs and effectively channels these metabolites. The residual structural framework of the cell nucleus, termed the nuclear protein matrix or "replisome," has been associated with newly synthesized DNA in regenerating rat liver and 3T3 fibroblasts (50, 51). The replitase described in our report could be the functional component of this DNA–membrane matrix in intact cells.

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