Metabolism of thymine nucleotides synthesized via the 'de novo' mechanism in normal, megaloblastic and methotrexate-treated human cells and in a lymphoblastoid cell line

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Human bone-marrow cells and lymphocytes were incubated with [³H]deoxyuridine (dU) to study the metabolism of thymine nucleotides labelled via the thymidylate synthase (5,10-methylenetetrahydrofolate:dUMP C-methyltransferase, EC 2.1.1.45) step of the 'de novo' biosynthetic pathway. (1) Continuous labelling with [³H]dU was used to compare incorporation of label into DNA with the specific radioactivities of thymine nucleotides separated by paper chromatography. (2) Cells were also labelled with [³H]dU at 13°C, and 'chased' in unlabelled medium at 37°C in order to quantify the proportion of thymine nucleotides incorporated into DNA and the proportion degraded. Only 40% of labelled thymine nucleotides were incorporated into lymphocyte DNA during a 'chase', whereas 100% were incorporated by MOLT 4 cells (a lymphoblastoid cell line of thymic origin, Thy-ALL line). Unincorporated nucleotides were rapidly degraded in lymphocytes, but degradative activity was very low in MOLT 4 cells. The results described here reinforce our previous conclusions [Taheri, Wickremasinghe & Hoffbrand (1981) *Biochem. J.* **194**, 451–461] that there is a single thymine nucleotide compartment in Thy-ALL cells, but at least two pools in lymphocytes and bone-marrow cells. This compartmentation of nucleotides in human cells is consistent with a model which proposes that deoxyribonucleotides are localized near replication forks by the activity of multienzyme complexes [Mathews, North & Reddy (1978) *Adv. Enz. Regul.* **17**, 133–156]. Our results also suggest that thymine nucleotides derived by the 'de novo' mechanism may be more highly localized than those derived by salvage. In cells from patients with megaloblastic anaemia owing to deficiency of vitamin B₁₂ or folate or in normal cells treated with methotrexate, there was a massive accumulation of labelled dUMP and decreased incorporation of label into DNA. There was no measurable incorporation of labelled deoxyuridine residues into DNA of megaloblastic cells, but deoxyuridine residues were detected in DNA of cells treated with methotrexate.

Megaloblastic anaemia commonly results from a deficiency of either folate or vitamin B₁₂. The biochemical and morphological abnormalities are thought to result from impaired DNA replication owing to decreased biosynthesis of dTTP (Metz *et al.*, 1968; Hoffbrand *et al.*, 1976; Ganeshaguru & Hoffbrand, 1978), which is one of the four proximate precursors of DNA (Baumann & Schindler, 1979; Baumann *et al.*, 1979). Although defects in several stages of DNA replication have been demonstrated in megaloblastic cells (Wickremasinghe & Hoffbrand, 1979, 1980*a,b*), the dTTP concentration in megaloblasts is similar to that in normal

Abbreviation used: Thy acute lymphoblastic leukaemia, acute lymphoblastic leukaemia of thymic origin.

cells (Hoffbrand *et al.*, 1974). This observation led us (Wickremasinghe & Hoffbrand, 1979, 1980*a,b*) and others (Mathews *et al.*, 1978) to propose that DNA precursors were functionally compartmentalized in these cells. It was suggested that a small, rapidly turning-over dTTP pool was highly localized near the DNA-replication machinery, while a larger, more diffuse pool which did not contribute to DNA replication was present. Thus changes in the small pool would affect DNA replication, but these changes might be obscured by the presence of the larger pool in measurement of the overall cell dTTP concentration.

In a previous study (Taheri *et al.*, 1981*b*) we labelled cells with [³H]dT and studied the fate of thymine

nucleotides labelled via thymidine kinase (ATP:thymidine 5'-phosphotransferase, EC 2.7.1.21), i.e. the so-called 'salvage' pathway (Kornberg, 1976). In phytohaemagglutinin-stimulated lymphocytes and in bone-marrow cells only a proportion (30–60%) of labelled thymine nucleotides were available for DNA synthesis, the rest being rapidly degraded and lost from the cell. In contrast, a lymphoblastoid-cell line from a patient with Thy acute lymphoblastic leukaemia used nearly 100% of its labelled thymine nucleotides for DNA replication.

In this paper we have extended these observations by using [^3H]dU to label thymine nucleotides via the thymidylate synthase (5,10-methylene-tetrahydrofolate:dUMP C-methyltransferase, EC 2.1.1.45) step, a component of the 'de novo' pathway of dTTP synthesis (Kornberg, 1976). The results reported here confirm our previous conclusions on functional compartmentation. In addition, they indicate that nucleotides derived via the 'de novo' pathway may be more closely localized near the precursor-synthesizing enzymes than are those derived by salvage. Finally, labelling with [^3H]dU also enabled us to demonstrate directly inhibition of thymidylate synthase in megaloblastic anaemia owing to deficiency of vitamin B₁₂ or folate, whereas previously this defect has also been studied indirectly by the 'dU suppression test' (Killman, 1964; Metz *et al.*, 1968; Das & Hoffbrand, 1970b; Van der Weyden *et al.*, 1973).

Materials and methods

Reagents

[5-Me- ^3H]Thymidine ([^3H]dT; 25 Ci/mmol) and deoxy[6- ^3H]uridine were from The Radiochemical Centre, Amersham, Bucks., U.K. Marker nucleosides and nucleotides, snake-venom phosphodiesterase (*Bothrops atrox*) and sodium dodecyl sulphate were supplied by Sigma, St. Louis, MO, U.S.A. The sources of other reagents have been described previously (Taheri *et al.*, 1981b).

Cell cultures and labelling

Lymphocytes were purified and cultured in the presence of phytohaemagglutinin (Taheri *et al.*, 1981b). Lymphocytes from patients with megaloblastic anaemia were cultured in medium 199 without folic acid. Folinic acid (30 $\mu\text{g}\cdot\text{ml}^{-1}$) and vitamin B₁₂ (10 $\mu\text{g}\cdot\text{ml}^{-1}$) were added to half of the cultures to provide 'control' lymphocytes (Wickremasinghe & Hoffbrand, 1979, 1980a,b; Das & Hoffbrand, 1970a,b).

Human bone marrow was obtained by aspiration from the iliac crest, suspended in Hanks' salts solution containing 20% autologous serum and used within 1 h for labelling experiments. Preincubation

for 1 h with vitamin B₁₂ (10 $\mu\text{g}\cdot\text{ml}^{-1}$) and folinic acid (30 $\mu\text{g}\cdot\text{ml}^{-1}$) was carried out on half the cells from megaloblastic marrows. MOLT 4 cells, an established cell line from a patient with Thy acute lymphoblastic leukaemia, were propagated in RPMI medium supplemented with 10% foetal-calf serum, penicillin (50 units $\cdot\text{ml}^{-1}$) and streptomycin (50 $\mu\text{g}\cdot\text{ml}^{-1}$).

Labelling with radioactive nucleosides, extraction of soluble nucleotides and DNA, and measurement of radioactivity were as previously described (Taheri *et al.*, 1981b), except that cells were concentrated to $2 \times 10^6\text{ml}^{-1}$ for labelling. [^3H]dT and [^3H]dU were added at final concentrations of 5 μCi (0.2 pmol) $\cdot\text{ml}^{-1}$.

Separation of dT and dU nucleosides and nucleotides

This was done by chromatography on Whatman no. 1 paper. The mobile phase consisted of isobutyric acid/conc. NH₃ (sp.gr. 0.880)/water (25:1:14, by vol.) containing 1 mM-EDTA. Marker nucleotides were located under u.v. light, the spots cut out and processed for scintillation counting of radioactivity (Taheri *et al.*, 1981b).

Extraction and enzymic hydrolysis of DNA

DNA was extracted from labelled cells by published procedures (Wickremasinghe & Hoffbrand, 1980a). The DNA was dissolved in 10 mM-NaCl and denatured by heating at 100°C for 5 min. Digestions were carried out in 20 mM-Tris/HCl (pH 8.8)/10 mM-MgCl₂, containing snake-venom phosphodiesterase (10 mg $\cdot\text{ml}^{-1}$) and DNA (about 1 mg $\cdot\text{ml}^{-1}$). The digest was chromatographed on Whatman no. 1 paper as described above. The predominant products were nucleosides, owing to the presence of 5'-nucleotidase in the venom preparation.

Results

Labelling of phytohaemagglutinin-stimulated lymphocytes with [^3H]dT or [^3H]dU

Initially we compared the time course of labelling of lymphocytes with [^3H]dT and [^3H]dU. Cells were concentrated to $2 \times 10^6\text{ml}^{-1}$ in fresh medium and labelled with either [^3H]dT or [^3H]dU. Incorporation of [^3H]dT into DNA became linear with respect to time after a brief lag (about 2 min). A longer lag (about 10 min) was evident when [^3H]dU was the precursor (Fig. 1). Nevertheless, once linear rates of labelling had been established, incorporation rates from the two precursors were very similar. If the lymphocytes had been pretreated for 1 h with 1 μM -methotrexate, DNA labelling from [^3H]dU was almost totally abolished (Fig. 1). This was associated with a massive intracellular accumulation of

Fig. 1. Labelling of lymphocytes with [³H]dT or [³H]dU. Phytohaemagglutinin-stimulated lymphocytes (2×10^6 cells $\cdot$ ml⁻¹) were labelled with either [³H]dT or [³H]dU (5μ Ci $\cdot$ ml⁻¹, 25 Ci $\cdot$ mmol⁻¹). Samples (1 ml) were withdrawn at the indicated times and processed for determination of radioactivity in DNA. Δ , [³H]dT; $\circ$, [³H]dU; $\bullet$, [³H]dU in the presence of 1μ M-methotrexate.

[³H]-dUMP (results not shown), as expected from the known action of methotrexate in inhibiting indirectly the methylation of dUMP to dTMP. RNA was not labelled by [³H]dU, since none of the acid-precipitable radioactivity in cells labelled with this precursor could be solubilized by alkali (results not shown).

Phytohaemagglutinin-stimulated lymphocytes from a patient with megaloblastic anaemia labelled with [³H]dU

In the untreated megaloblastic lymphocytes (Fig. 2a) a large build-up of [³H]dUMP was evident. This was dramatically decreased if the lymphocytes had been cultured in the presence of vitamin B₁₂ and folinic acid (Fig. 2b). Since the amounts of radiolabelled precursors used in these studies were so small as to cause undetectable changes in the intracellular dTTP pools (Taheri *et al.*, 1981b), we assumed that the radiolabel in a given DNA precursor was proportional to the overall specific radioactivity of that precursor. As expected, the rate of labelling of dTMP was lower in the untreated cells than in the vitamin-treated cells. Surprisingly, the rate of DNA labelling was only slightly lower in the untreated cells, although the untreated cells did

display a longer lag phase before a linear rate of DNA labelling was established.

In both Figs. 2(a) and 2(b), many of the nucleotide pools exhibited a biphasic labelling pattern, with a rapid initial burst of labelling, followed by a slower increase to a plateau (e.g. the [³H]dUMP pool in Fig. 2a and the dTMP, dTDP and dTTP pools in Fig. 2b). The plateau presumably results from a balance in the rates of synthesis and of metabolism of individual radiolabelled intermediates. The labelling of dTTP in Fig. 2(b) was of particular interest. The initial rapid rise (up to 2 min) in the specific radioactivity of dTTP correlated well with the lag phase in DNA labelling. The subsequent slower rise (up to 30 min) occurred during a period when DNA labelling was linear with respect to time. Therefore it was evident that the second phase in the increase of dTTP specific radioactivity was due to the labelling of a portion of the cellular dTTP that was not available for DNA synthesis.

Bone-marrow cells from a patient with megaloblastic anaemia labelled with [³H]dU at 37°C

The rate of incorporation of label from [³H]dU into DNA was markedly slower in the untreated marrow (Fig. 3a) than in the vitamin-treated cells from the same patient (Fig. 3b). In the vitamin-treated cells a brief lag (about 2 min) was followed by linear incorporation. In the untreated cells the rate never became linear, indicating that the label from [³H]dU did not equilibrate with the precursor dTTP pool during the course of the incubation. This was reflected by the massive accumulation of labelled dUMP in the untreated marrow. On the other hand, label in dUMP was virtually undetectable in vitamin-treated marrow. Furthermore, the accumulation of label in dTTP was very slow in untreated marrow. In the treated marrow the labelling of dTTP was biphasic, with a rapid rise in the first 1 min, followed by a slower increase up to 20 min. As in the experiment with lymphocytes (Fig. 2b), the initial rapid burst corresponded temporally to the lag in DNA labelling. Although the radiolabel in dTTP in the untreated cells was nearly equal to that in the treated cells at 60 min, the rate of DNA labelling in the untreated cells was only 28% of that in the treated cells at that time. It was therefore evident that the rate of DNA labelling was not dependent on the presence of labelled dTTP in the cells, but on some other factor such as the rate of its synthesis. Finally, the amounts of labelled dUDP and dUTP were elevated in the untreated relative to the treated cells. An identical experiment carried out on marrow cells from a normal donor (results not shown) gave results similar to those for the vitamin-treated marrow shown here. Addition of vitamins to normal marrow produced no changes in the

Fig. 2. Labelling of megaloblastic lymphocytes with $[^3\text{H}]\text{dU}$

Phytohaemagglutinin-stimulated lymphocytes from a patient with megaloblastic anaemia ($2 \times 10^6 \text{ cells} \cdot \text{ml}^{-1}$) were labelled with $[^3\text{H}]\text{dU}$ ($5 \mu\text{Ci} \cdot \text{ml}^{-1}$). Samples (1 ml) were withdrawn at the indicated times and processed for determination of radioactivity in DNA (Δ) and in nucleotides: $\circ$, total nucleotides; $\blacktriangle$, dTMP; $\square$, dTDP; $\bullet$, dTTP; $\blacksquare$, dUMP; ∇ , dUDP; $\ominus$, dUTP. For clarity, some of the data are presented in insets in this and following Figures (note the different scale). (a) No additions; (b) cells cultured in the presence of folinic acid ($30 \mu\text{g} \cdot \text{ml}^{-1}$) and vitamin B₁₂ ($10 \mu\text{g} \cdot \text{ml}^{-1}$).

rate of DNA labelling and only small changes in the accumulation of labelled dUMP.

Cultured *T* lymphoblasts (MOLT 4) labelled with $[^3\text{H}]\text{dU}$ with and without pre-treatment with methotrexate

In cells labelled in the absence of the drug, a linear rate of DNA labelling was established after a brief (about 1 min) lag (Fig. 4a). The amount of $[^3\text{H}]\text{-dUMP}$ was negligible. The pools of dTDP and dTTP reached a plateau specific radioactivity in a single rapid burst of 1–2 min duration. This was in contrast with the biphasic labelling pattern of lymphocytes (Fig. 2). This was compatible with a single compartment of dTTP derived *de novo* in MOLT 4 cells. In methotrexate-treated cells (Fig. 4b) a massive biphasic accumulation of $[^3\text{H}]\text{dUMP}$ was seen. Labelled dUDP and dUTP were also increased.

DNA labelling was greatly decreased. However, some labelling of dTTP did occur. At 60 min the label in dTTP reached 57% of that in control cells. Nevertheless DNA labelling rate was only 8% of that in the control cells.

Preliminary incubation of lymphocytes with $[^3\text{H}]\text{dU}$ at 13°C followed by a chase at 37°C

(a) *Normal lymphocytes.* In a previous paper (Taheri *et al.*, 1981b) we demonstrated that only a proportion (40–60%) of thymine nucleotides labelled at 13°C in lymphocytes or bone-marrow cells incubated with $[^3\text{H}]\text{dT}$ was incorporated into DNA, the rest being degraded. Since thymine nucleotides labelled from $[^3\text{H}]\text{dT}$ are derived via the salvage pathway, it was decided to determine whether thymine nucleotides synthesized via thymidylate

Fig. 3. Labelling of megaloblastic bone marrow with $[^3\text{H}]\text{dU}$

Bone-marrow cells from a patient with megaloblastic anaemia ($2 \times 10^6 \text{ cells} \cdot \text{ml}^{-1}$) were labelled with $[^3\text{H}]\text{dU}$ ($5 \mu\text{Ci} \cdot \text{ml}^{-1}$). Samples (1 ml) were withdrawn at the indicated times and processed for the determination of radioactivity in DNA (Δ) and in nucleotides: $\circ$, total nucleotides; $\blacktriangle$, dTMP; $\square$, dTDP; $\bullet$, dTTP; $\blacksquare$, dUMP; ∇ , dUDP; $\odot$, dUTP. (a) No additions; (b) cells preincubated for 1 h with folinic acid ($30 \mu\text{g} \cdot \text{ml}^{-1}$) and vitamin B₁₂ ($10 \mu\text{g} \cdot \text{ml}^{-1}$).

synthase in the 'de novo' mechanism also showed functional compartmentation. Phytohaemagglutinin-stimulated lymphocytes (72 h cultures) were concentrated to $2 \times 10^6 \text{ cells} \cdot \text{ml}^{-1}$ in fresh medium, cooled to 13°C and labelled with $[^3\text{H}]\text{dU}$ for 20 min. They were then collected by centrifugation at $700g$ at 13°C , for 5 min, and resuspended quickly in medium prewarmed to 37°C and containing 20 nM unlabelled dU. Samples were removed for determination of radiolabel in DNA and in nucleotides. This protocol was adopted because we found in preliminary experiments that incorporation of radiolabel from $[^3\text{H}]\text{dU}$ into DNA was negligible at 13°C , but maximal labelling of nucleotides took place. This permitted us to quantify clearly the proportion of labelled thymine nucleotide entering DNA during the chase at 37°C . Incubation at 13°C does not impair the capacity of cells to synthesize DNA on subsequent shift-up to 37°C (Taheri *et al.*, 1981b).

Fig. 5(a) shows the results of an experiment carried out by this protocol. The label in dTDP and

dTTP showed transient rises in the first 1 min of the incubation at 37°C and then fell. The label in dTMP fell steadily during this period. There was insufficient radiolabel in dTTP alone at the start of the incubation at 37°C to account for the label incorporated into DNA. Therefore at least a part of the dTMP and/or dTDP must have been phosphorylated to dTTP. However, the total label incorporated into DNA (68000 d.p.m.) was only a fraction (40%) of the total label lost from thymine nucleotides. Thus a proportion of thymine nucleotides derived via the 'de novo' pathway are also subject to degradation, as are those derived by salvage (Taheri *et al.*, 1981b). The label in the dUMP pool (50000 d.p.m.) fell rapidly to 10000 d.p.m. within 1 min after shifting the cells to 37°C . Since incorporation into DNA together with the transient rises in thymine nucleotide pools in the same time interval did not account for the loss of 40000 d.p.m. from dUMP, it seems likely that most of the accumulated dUMP was also degraded.

To confirm that loss of label from thymine

Fig. 4. *MOLT 4* cells labelled with $[^3\text{H}]\text{dU}$

Cultured T lymphoblasts (*MOLT 4*) were labelled with $[^3\text{H}]\text{dU}$ ($5 \mu\text{Ci} \cdot \text{ml}^{-1}$). Samples (1 ml) were withdrawn at the indicated times and processed for the determination of radioactivity in DNA (Δ) and in nucleotides: $\bigcirc$, total nucleotides; $\blacktriangle$, dTMP; $\square$, dTDP; $\bullet$, dTTP; $\blacksquare$, dUMP; ∇ , dUDP; $\odot$, dUTP. (a) No additions; (b) cells pre-treated for 1 h with $1 \mu\text{M}$ -methotrexate.

Fig. 5. Labelling of lymphocytes with $[^3\text{H}]\text{dU}$ at 13°C followed by 'chase' at 37°C

Phytohaemagglutinin-stimulated lymphocytes ($2 \times 10^6 \text{ cells} \cdot \text{ml}^{-1}$) were labelled with $[^3\text{H}]\text{dU}$ ($5 \mu\text{Ci} \cdot \text{ml}^{-1}$) for 20 min at 13°C . They were collected by centrifugation and resuspended at the same concentration in medium pre-warmed to 37°C and containing 80 nM -unlabelled dU. Samples (1 ml) were withdrawn at the indicated times for determination of radioactivity in DNA (Δ) and in nucleotides: $\bigcirc$, total nucleotides; $\blacktriangle$, dTMP; $\square$, dTDP; $\bullet$, dTTP; $\blacksquare$, dUMP; ∇ , dUDP; $\odot$, dUTP. (a) No additions; (b) cells preincubated for 1 h with $10 \mu\text{M}$ -cytosine arabinoside.

Fig. 6. Labelling of megaloblastic lymphocytes with $[^3\text{H}]\text{dU}$ at 13°C followed by 'chase' at 37°C

Phytohaemagglutinin-stimulated lymphocytes from a patient with megaloblastic anaemia (2×10^6 cells $\cdot \text{ml}^{-1}$) were labelled with $[^3\text{H}]\text{dU}$ ($5 \mu\text{Ci} \cdot \text{ml}^{-1}$) at 20 min at 13°C . They were collected by centrifugation and resuspended at the same concentration in medium prewarmed to 37°C and containing 80 nM unlabelled dU. Samples (1 ml) were withdrawn at the indicated times for determination of radioactivity in DNA (Δ) and in nucleotides: $\circ$, total nucleotides; $\blacktriangle$, dTMP; $\square$, dTDP; $\bullet$, dTTP; $\blacksquare$, dUMP; ∇ , dUDP; $\ominus$, dUTP. (a) No additions; (b) cells cultured in the presence of folinic acid ($30 \mu\text{g} \cdot \text{ml}^{-1}$) and vitamin B_{12} ($10 \mu\text{g} \cdot \text{ml}^{-1}$).

nucleotide pools could occur independently of DNA synthesis, the experiment was repeated in the presence of the replication inhibitor cytosine arabinoside ($1\text{-}\beta\text{-D-arabinofuranosylcytosine}$). The distribution of label in lymphocytes incubated with $[^3\text{H}]\text{dU}$ at 13°C in the presence of the drug (Fig. 5b) was very similar to that in cells labelled in the absence of inhibitor (Fig. 5a). When the cells were shifted to 37°C in unlabelled medium, almost complete loss of label from all thymine nucleotides occurred. However, the label in dTDP and dTTP showed transient increases before falling. A total of 95 000 d.p.m. was lost from thymine nucleotides, whereas only 4000 d.p.m. (4.2%) of this was incorporated into DNA. This experiment confirms the existence of a degradative pathway available to thymine nucleotides derived via the thymidylate synthase step.

(b) *Lymphocytes from a patient with megaloblastic anaemia.* Lymphocytes from a patient with megaloblastic anaemia were cultured in the absence (Fig. 6a) or presence (Fig. 6b) of vitamin B_{12} and folinic acid, labelled with $[^3\text{H}]\text{dU}$ at 13°C and chased at 37°C . At the beginning of the chase the untreated cells contained a large amount of $[^3\text{H}]\text{dUMP}$ (Fig. 6a), whereas the amount of this compound was very low in vitamin-treated cells

(Fig. 6b). Incorporation of label into DNA in untreated cells during the chase was nearly twice that in the treated cells. In the treated cells 42% of thymine nucleotides were incorporated into DNA. Once again, transient rises in labelled dTDP and dTTP in the early part of the chase reflected synthesis of these compounds from $[^3\text{H}]\text{dTMP}$. In untreated cells the incorporation of label into DNA (64 000 d.p.m.) matched the loss of label from thymine nucleotide (56 000 d.p.m.) within experimental error. It was unlikely that a significant amount of labelled thymine nucleotide was synthesized from $[^3\text{H}]\text{dUMP}$ during the chase, because no transient increase in labelled dTMP was seen at any time during the chase. Furthermore, the decrease in labelled dTMP during the first 4 min of chase (19 000 d.p.m.) was exactly equal to the increase in label in dTDP, dTTP and DNA taken together, a relationship that would not be expected if significant amounts of $[^3\text{H}]\text{dTMP}$ were synthesized from $[^3\text{H}]\text{dUMP}$ during the chase. The loss of 62 000 d.p.m. from dUMP during the first 4 min of chase was probably due to its degradation. Thus, in accordance with our previous observations on the fate of salvage-derived thymine nucleotides in megaloblastic lymphocytes (Taheri *et al.*, 1981b), the present observations confirm that utilization of

Fig. 7. Labelling of MOLT 4 cells with $[^3\text{H}]\text{dU}$ at 13°C followed by 'chase' at 37°C

MOLT 4 cells (2×10^6 cells $\cdot \text{ml}^{-1}$) were labelled with $[^3\text{H}]\text{dU}$ ($5 \mu\text{Ci} \cdot \text{ml}^{-1}$) for 20 min at 13°C . They were collected by centrifugation and resuspended at the same concentration in medium pre-warmed to 37°C and containing 80 nM-unlabelled dU. Samples (1 ml) were withdrawn at the indicated times for determination of radioactivity in DNA (Δ) and in nucleotides: $\circ$, total nucleotides; $\blacktriangle$, dTMP; $\square$, dTDP; $\bullet$, dTTP; $\blacksquare$, dUMP; ∇ , dUDP; $\odot$, dUTP.

thymine nucleotides is more efficient in untreated megaloblastic cells than in vitamin-treated cells. In further support of this hypothesis, the transient increases in labelled dTDP and dTTP during the chase were more pronounced in the untreated cells (cf. Figs. 6a and 6b), indicating that a greater proportion of dTMP entered DNA (via dTDP and dTTP) in these cells than in the treated cells.

(c) *MOLT 4 cells.* MOLT 4 cells were labelled with $[^3\text{H}]\text{dU}$ at 13°C and chased at 37°C (Fig. 7). In these cells the fall in total labelled nucleotides ($110000 \text{ d.p.m.}/10^6$ cells) matched almost exactly the incorporation into DNA (115000 d.p.m.) during the chase. In contrast with the situation in lymphocytes, all of the labelled nucleotides in MOLT 4 cells were used for DNA synthesis and were not degraded. This was confirmed by repeating the

experiment in the presence of cytosine arabinoside. Incorporation of label into DNA was completely suppressed during the chase. The labelled nucleotides were almost completely conserved during the chase, only 9% being degraded in 1 h (results not shown).

Are deoxyuridine nucleotides incorporated into DNA?

Finally, we investigated the possibility that under conditions of blocked conversion of dUMP into dTMP, the abnormal accumulation of dUTP might result in incorporation of some deoxyuridine residues into DNA. We examined by paper chromatography phosphodiesterase digests of DNA purified from cells labelled with $[^3\text{H}]\text{dU}$ under various conditions (Table 1). Small amounts of radioactivity (2–5% of total) were found in the deoxyuridine spot in lymphocytes or bone-marrow cells from two patients with megaloblastic anaemia irrespective of whether the cells had been treated with vitamins or not (lines 4–11). However, this was due to trailing of label from thymidine residues, since a similar proportion of label was found in the dU nucleotide position when lymphocytes were labelled with $[^3\text{H}]\text{dT}$ (line 1). A significantly increased proportion (19%) of label was found in dU residues in MOLT 4 cells that had been pre-treated with methotrexate (cf. lines 12 and 13, lines 14 and 15).

Discussion

Our previous studies (Taheri *et al.*, 1981b) on the labelling of eukaryotic cells with $[^3\text{H}]\text{thymidine}$ led us to conclude that thymine nucleotides derived via the salvage pathway were subjected to functional compartmentation in human lymphocytes and bone-marrow cells, but not in a permanent lymphoblastoid cell line (HPB-ALL). In the present work we extended these observations to thymine nucleotides labelled by $[^3\text{H}]\text{deoxyuridine}$ via the thymidylate synthetase step, a component of the 'de novo' pathway of thymine nucleotide biosynthesis.

Initially we compared the labelling of phytohaemagglutinin-stimulated lymphocytes with $[^3\text{H}]\text{dT}$ and $[^3\text{H}]\text{dU}$. The lag phase in DNA labelling was longer when $[^3\text{H}]\text{dU}$ was the precursor, but once linear labelling rates had been established incorporation rates from either precursor were nearly identical. The longer lag phase in $[^3\text{H}]\text{dU}$ labelling reflects the longer time required for the equilibration of the precursor dTTP pool with exogenous $[^3\text{H}]\text{dU}$, since the biosynthetic route from dU to dTTP involves more steps than that from dT to dTTP.

Conditions that interfered with the thymidylate synthase step resulted in large intracellular accumulations of $[^3\text{H}]\text{dUMP}$, compared with appropriate controls. This was seen in cells treated with

Table 1. Analysis of labelled residues in DNA of cells incubated with [^3H]dT or [^3H]dU

Cells were labelled as indicated, the DNA extracted, hydrolysed with snake-venom phosphodiesterase and chromatographed as described in the Materials and methods section. Labelled nucleosides were supplied at $5\mu\text{Ci}\cdot\text{ml}^{-1}$. Concentrations of other additions were: methotrexate, $1\mu\text{M}$, folic acid, $30\mu\text{g}\cdot\text{ml}^{-1}$; vitamin B_{12} , $10\mu\text{g}\cdot\text{ml}^{-1}$.

Cells used	Labelled precursors	Additions	Total radioactivity analysed (d.p.m.)	Percentage of label recovered in	
				dT nucleotides	dU nucleotides
Normal lymphocytes	1 [^3H]dT	None	74 200	98.4	1.6
	2 [^3H]dU	None	28 490	98.7	1.3
	3 [^3H]dU	Methotrexate	2000	89.5	10.5
Megaloblastic-anaemia patient 1: (a) Bone marrow	4 [^3H]dU	None	12 530	97	3
	5 [^3H]dU	Folic acid, vitamin B_{12}	21 820	95	5
	6 [^3H]dU	None	102 300	98	2
	7 [^3H]dU	Folic acid, vitamin B_{12}	251 250	98.5	1.5
Megaloblastic-anaemia patient 2: (a) Bone marrow	8 [^3H]dU	None	23 750	98	2
	9 [^3H]dU	Folic acid, vitamin B_{12}	36 960	98	2
	10 [^3H]dU	None	118 580	97	3
	11 [^3H]dU	Folic acid, vitamin B_{12}	226 670	98	2
T lymphoblastoid cell line (MOLT 4)	12 [^3H]dU	None	38 340	98	3
	13 [^3H]dU	Methotrexate	1670	81	19
	14 [^3H]dU	None	127 800	99	1
	15 [^3H]dU	Methotrexate	8350	81	19

the folate antagonist methotrexate, and in lymphocytes (Fig. 2) and bone-marrow cells (Fig. 3) from patients with megaloblastic anaemia. All previous studies have only shown the block in thymidylate synthase in megaloblastic anaemia by indirect techniques. The label in dUDP and dUTP was also increased in megaloblastic and methotrexate-treated cells. Incorporation of label from [^3H]dU into DNA was decreased as expected in methotrexate-treated cells (Figs. 1 and 4) and in megaloblastic marrow cells (Fig. 3). However, despite the accumulation of [^3H]dUMP in megaloblastic lymphocytes, labelling of DNA was only slightly depressed compared with that in vitamin-treated controls (Fig. 2).

Studies on the incorporation of [^3H]dU into the DNA of human marrow cells have also been reported by Van der Weyden (1979). However, no enhancement of incorporation into megaloblastic marrow cells was caused by treatment with folic acid or vitamin B_{12} . In our hands [^3H]dU incorporation into megaloblastic marrow from 16 patients has invariably been increased by treatment with appropriate vitamins (Taheri *et al.*, 1981a).

Interestingly, the overall specific radioactivity of cellular dTTP did not determine its rate of incorporation into DNA. For example, in the experiment shown in Fig. 3 the overall specific radioactivity of dTTP at 60 min was nearly equal in the untreated

and vitamin-treated megaloblastic marrow cells, since the size of the dTTP pool in megaloblastic anaemia is not decreased (Hoffbrand *et al.*, 1974). Nevertheless, the rate of DNA labelling in untreated cells was only 28% of that in the treated cells. Thus, even though labelled [^3H]dTTP synthesized by the thymidylate synthase step could accumulate to nearly equal specific radioactivities in cells in which this reaction was inhibited as in uninhibited cells, this dTTP was less available for incorporation into DNA in the inhibited cells. These observations are in accord with some form of compartmentation of DNA precursors, as previously suggested by others for prokaryotic (Werner, 1971; Pato, 1979) and eukaryotic (Baumunk & Friedman, 1971; Fridland, 1973; Kuebbing & Werner, 1975) systems.

The biphasic labelling of cellular dTTP by [^3H]dU reinforces the conclusion that DNA precursors are compartmentalized in some cell types. As pointed out by others (Werner, 1971; Fridland, 1973) the specific radioactivity of the dTTP pool which serves as the proximate precursor for DNA synthesis must attain its maximum value before a linear rate of DNA labelling is established. In lymphocytes (Fig. 2) and in bone-marrow cells (Fig. 3) the increase in specific radioactivity of dTTP was distinctly biphasic. An initial rapid increase in [^3H]dTTP was complete at the time DNA labelling became linear.

A subsequent slower increase in [^3H]TTP was evident, although the rate of DNA labelling remained constant. Our interpretation of these observations is that the initial burst represents the labelling of rapidly turning-over dTTP pool, which is utilized for DNA synthesis, whereas the slower phase results from the labelling of a dTTP pool unavailable for replication. Similar conclusions were drawn by Fridland (1973), who labelled human lymphoblasts with both [^3H]dT and [^3H]dU. However, his results did not show the markedly biphasic dTTP labelling seen in the present work. In our hands, a lymphoblastoid-cell line (MOLT 4) showed only a single burst of dTTP labelling, correlating temporally with the establishment of linear DNA labelling (Fig. 4a). These cells appeared to contain a single dTTP pool under normal circumstances, a conclusion confirmed by the chase experiments discussed below.

Compartmentation of thymine nucleotides was investigated further by labelling cells with [^3H]dU at 13°C and chasing in the presence of unlabelled dU at 37°C. In lymphocytes (Fig. 5a) only 40% of labelled thymine nucleotides were incorporated into DNA during the chase, the rest being degraded to a form lost from the cell. Similar observations have been made with cells labelled with [^3H]dT. However, in contrast with the behaviour of thymine nucleotides labelled by [^3H]dT, transient increases in labelled dTDP and dTTP occurred during the chase of cells labelled with [^3H]dU. In this context it is of interest to consider a mechanism proposed by Mathews *et al.* (1978) for the compartmentation of DNA precursors during replication of the coliphage T4. This mechanism has subsequently been extended to eukaryotic cells (Reddy & Pardee, 1980). According to this model DNA precursors are synthesized by a kinetically coupled enzyme complex located near the replication fork. In this way a higher DNA-precursor concentration can be maintained at the fork than would be possible if they were synthesized by a soluble enzyme system. Our results indicate that [^3H]dTMP derived from [^3H]dUMP via the thymidylate synthase step is more closely localized near the multienzyme complex than is [^3H]dTMP derived from [^3H]dT by the salvage pathway. We base this conclusion on two main lines of evidence. Firstly, the transient increases in labelled dTDP and dTTP that were seen during a chase of cells labelled with [^3H]dU (Figs. 5 and 6) were never seen in cells labelled with [^3H]dT (Taheri *et al.*, 1981b). Secondly, vitamin-deficient lymphocytes incorporated into DNA all labelled thymine nucleotide derived from [^3H]dU (Fig. 6), whereas only 50–60% was incorporated by similar cells which were labelled with [^3H]dT (Taheri *et al.*, 1981b). The complete incorporation of all labelled thymine nucleotides by MOLT 4 cells during a chase

(Fig. 7) indicates a high degree of localization of DNA precursors in these cells, which was probably responsible for the detection of a single dTTP compartment.

The operation of pathways for the degradation of thymine nucleotides was confirmed in lymphocytes by repeating the chase experiment in the presence of the replication inhibitor cytosine arabinoside (Fig. 5b). All DNA precursors labelled by [^3H]dU were degraded and lost from the cells, even though DNA synthesis was negligible. However, transient increases in labelled dTDP and dTTP were again evident, indicating that some phosphorylation of dTMP through dTDP to dTTP occurred before nucleotide degradation. In contrast, when MOLT 4 cells were labelled with [^3H]dU at 13°C and chased in the presence of cytosine arabinoside, the labelled nucleotides were almost completely stable in the absence of incorporation into DNA. This experiment confirmed the absence of a degradative route for thymine nucleotides in these cells.

Untreated megaloblastic lymphocytes incorporated labelled thymine nucleotides into DNA with an efficiency of 100% during the course of a chase experiment. Corresponding vitamin-treated cells only incorporated 40% of labelled nucleotide into DNA. These observations are consistent with our earlier observations (Taheri *et al.*, 1981b), which suggested that the efficiency of a precursor-synthesizing multienzyme complex could adapt to the availability of substrates, becoming more efficient when the supply of substrates becomes limited, e.g. when deficiency of vitamin B₁₂ or folate decreases thymidylate synthase activity.

Prokaryotic and eukaryotic cells possess mechanisms for excluding uridine residues from DNA. These comprise a dUTPase, which breaks down dUTP to dUMP (Tye *et al.*, 1977; Grafstrom *et al.*, 1978; Wist, 1979) and an uracil *N*-glycosidase, which excises uracil from DNA (Wist *et al.*, 1978; Grafstrom *et al.*, 1978; Caradonna & Cheng, 1980). However, since we have demonstrated increased dUTP accumulation under conditions of thymidylate synthase inhibition, it was of interest to determine whether this led to an increased accumulation of uridine nucleotides in DNA. We analysed venom phosphodiesterase digests of DNA from cells labelled with [^3H]dU under various conditions (Table 1). Untreated bone marrow or lymphocytes from two patients with megaloblastic anaemia did not show an increase in [^3H]dUMP residues compared with vitamin-treated controls. However, DNA of cells labelled with [^3H]dU in the presence of methotrexate contained a significantly increased ratio of [^3H]dUMP to [^3H]dTMP residues. Presumably the acute inhibition of thymidylate synthase caused by this drug led to accumulation of amounts of dUTP which overwhelmed the exclusion mechanisms. This

facet of methotrexate action on cells may play a role in its cytotoxic action.

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