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Alternative metabolic fates of thymine nucleotides in human cells

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Three types of experiments have been used to study the metabolism of thymine nucleotides by human cells. (1) Cells were labelled continuously with [^{3}H]thymidine and the incorporation of label into DNA compared with the specific radioactivities of pools of individual thymine nucleotides separated by chromatography on polyethyleneimine-cellulose. (2) Cellular thymine nucleotides were labelled with [^{3}H]thymidine at 13°C, followed by incubation at 37°C in unlabelled medium. Incorporation of label into DNA and loss of label from the nucleotide pools were monitored during the 'chase' period at 37°C. (3) The experiments described in (2) above were repeated in the presence of the DNA-synthesis inhibitor cytosine arabinoside, in order to demonstrate more clearly and to quantify degradative pathways for thymine nucleotides. In phytohaemagglutinin-stimulated lymphocytes and in bone-marrow cells, only a proportion (25–60%) of labelled thymine nucleotide was incorporated into DNA, the rest being rapidly degraded and lost from the cell. In contrast, an established cell line (HPB-ALL) from a patient with acute lymphoblastic leukaemia of thymic origin incorporated 100% of its exogenously labelled thymine nucleotides into DNA. These results indicated that alternative metabolic routes are open to thymine nucleotides in human cells. In lymphocytes from patients with megaloblastic anaemia and in normal lymphocytes treated with methotrexate, the utilization of labelled thymine nucleotides for DNA synthesis was more efficient than in controls. These results offer an explanation for the observation of a normal pool of thymidine triphosphate in the cells of patients with untreated megaloblastic anaemia even though the amount of this compound available for DNA synthesis appears to be decreased.

A large body of evidence summarized by Kornberg (1976) suggests that dNTP species are the immediate precursors for DNA biosynthesis in mammalian and other cells. In bacterial systems these precursors are subjected to some form of compartmentation (Werner, 1971*a*). Reddy & Matthews (1978) have demonstrated that a multienzyme complex of precursor-synthesizing enzymes located near the replication fork of the coliphage T4 maintains a high local concentration of dNTP species in this area. dNTP pools may also be functionally compartmentalized in eukaryotic cells (Fridland, 1973*a*; Baumunk & Friedman, 1971; Kuebbing & Werner, 1975). However, the evidence for this is based on experiments with established cell lines.

Our interest in dNTP compartmentation stemmed from our previous observations on bone-marrow cells and phytohaemagglutinin-stimulated lymphocytes from patients with megaloblastic anaemia. Although there is good evidence that the biosynthesis of dTTP is impaired in cells from patients with megaloblastic anaemia due to either folate or vitamin B_{12} deficiency (Metz *et al.*, 1968; Hoffbrand *et al.*, 1976; Ganeshaguru & Hoffbrand, 1978), direct measurement of dNTP pools showed that these were essentially normal (Hoffbrand *et al.*, 1974). Nevertheless, several stages in DNA replication have also been shown to be impaired in these cells (Wickremasinghe & Hoffbrand, 1979, 1980*a*,*b*), suggesting deoxyribonucleotide starvation. It therefore seemed possible that alternative routes for deoxyribonucleotide metabolism, in addition to incorporation into DNA, might exist, accounting for these apparently conflicting observations. More specifically, we have proposed (Wickremasinghe & Hoffbrand, 1979, 1980*a*) that if only a small proportion of cellular dTTP was available for DNA replication, a decrease of this pool in megaloblastic anaemia may be masked by the

Abbreviations used: Thy-AL leukaemia, acute lymphoblastic leukaemia of thymic origin; AraC, cytosine arabinoside; butyl-PBD, 5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole.

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presence of a larger pool that was not available for DNA synthesis. The experiments described in the present paper demonstrate that only a proportion of thymine nucleotides are indeed used for DNA synthesis in human phytohaemagglutinin-stimulated lymphocytes and bone-marrow cells. In contrast, thymine nucleotides are incorporated into DNA with almost complete efficiency by an established cell line from a case of Thy-AL leukaemia.

Experimental

Reagents

[5-*Me*-^{3}H]thymidine (hereafter called [^{3}H]dT; 68.3 Ci/mmol) was purchased from ICN Pharmaceuticals Inc., Irvine, CA, U.S.A. Medium TC 199 and phytohaemagglutinin were from Wellcome Research Laboratories, Beckenham, Kent, U.K. RPMI 1640 medium was purchased from Gibco, Paisley, Renfrewshire, Scotland, U.K. Penicillin, streptomycin and L-glutamine were from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K. AraC was supplied by Upjohn, Crawley, Sussex, U.K. Polyethyleneimine-cellulose plates (Schleicher and Schüll) were purchased from Anderman and Co., East Molesey, Surrey, U.K. Triosil was from Nyegaard and Co., Oslo, Norway, and Ficoll from Pharmacia Fine Chemicals, Uppsala, Sweden.

Lymphocyte culture

Lymphocytes were purified from heparinized venous blood, obtained (with their informed consent) from normal healthy adult donors, by sedimentation through a Triosil/Ficoll gradient (Böyum, 1968) and cultured at an initial concentration of 5×10^5 cells·ml^{-1} in medium 199 in the presence of phytohaemagglutinin and 20% autologous serum (Das & Hoffbrand, 1970*b*). They were utilized for labelling experiments at 72 h of culture. Lymphocytes from patients with megaloblastic anaemia were cultured in medium 199 without folic acid or thymine. Folinic acid (30 μg·ml^{-1}) and cyanocobalamin (10 μg·ml^{-1}) were added to one half of the cultures before haemagglutinin stimulation in order to provide 'control' lymphocytes (Wickremasinghe & Hoffbrand, 1979, 1980*a*,*b*; Das & Hoffbrand, 1970*a*,*b*).

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

HPB-ALL cells, an established Thy-AL leukaemia, were obtained from the Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, London W.C.1, U.K., and maintained in RPMI medium containing 10% (v/v) foetal-calf serum, penicillin (50 units·ml^{-1}) and streptomycin (50 μg·ml^{-1}).

Labelling experiments

Cells were concentrated by centrifugation and resuspended to 5×10^6·ml^{-1} in their original growth medium. [^{3}H]dT was added to a final concentration of 5 μCi·ml^{-1} (73 nM) and portions were taken at intervals during incubation at 37°C (lymphocytes and bone marrow) or 22°C (HPB-ALL). Labelling was terminated by pipetting 1 ml portions of cell suspension into 4 ml of ice-cold phosphate-buffered saline (75 mM-NaCl/75 mM-sodium phosphate, pH 7.4) containing 600 nM unlabelled dT. The cell samples were centrifuged at 500 *g*, washed once with phosphate-buffered saline and nucleotides were extracted overnight by addition to the cell pellet of 1 ml of 60% methanol at −20°C (Lindberg & Skoog, 1970). The samples were centrifuged and the supernatant freeze-dried. The freeze-dried material was used to determine radioactivity in thymine nucleotides as described below.

The pellet from the methanol extract was washed once with cold 0.5 M-$HClO_4$ and hydrolysed for 20 min at 80°C in 0.5 ml of the same solution. Incorporation of [^{3}H]dT into DNA was determined by counting for radioactivity 100 μl portions of the hydrolysate in 10 ml of scintillation fluid (8 g of butyl-PBD, 80 g of naphthalene, 400 ml of 3-ethoxyethanol and 600 ml of toluene).

Separation of thymine nucleotides by polyethyleneimine-cellulose chromatography

The freeze-dried methanol extracts from [^{3}H]-dT-labelled cells were dissolved in 50 μl of a solution 1 mM in each of dT, dTMP, dTDP and dTTP. Samples (12.5 μl) were chromatographed on polyethyleneimine-cellulose plates, with 1 M-LiCl as solvent. Positions of the marker nucleotides were located under u.v. light (248 nm), and the spots cut out and extracted overnight with 5 ml of 4 M-NH_3. A portion (4 ml) of each extract was transferred to a scintillation vial, dried, dissolved in 100 μl of water and counted for radioactivity in 10 ml of scintillation fluid. Recovery of input label was 50%. The vials were counted for radioactivity in a LKB Wallac scintillation counter (LKB Wallac, Bromma, Sweden) programmed to convert counting data into d.p.m. by the external-standard method.

Other methods

dTTP concentration was measured in methanol extracts of cells (Lindberg & Skoog, 1970) and DNA was determined by the diphenylamine method (Burton, 1956), with calf thymus DNA as standard.

Results

Phytohaemagglutinin-stimulated lymphocytes labelled with [^{3}H]dT at 37°C

Initially lymphocytes were labelled with [^{3}H]dT and incorporation of label into DNA and into nucleotides was monitored (Fig. 1*a*). The rate of [^{3}H]dT incorporation into DNA increased up to 4 min and remained nearly constant between 4 and 15 min. The rate of DNA labelling subsequently fell, probably due to destruction of [^{3}H]dT in the medium (see below). The label in the dTTP pool showed an initial rapid increase up to 3–4 min, followed by a more gradual rise that continued until the termination of the experiment at 30 min. We have determined by direct assay of the dTTP pool (Lindberg & Skoog, 1970) that the concentration of [^{3}H]dT (73 nM) added in this experiment did not significantly increase the dTTP concentration in the lymphocytes (8–9 pmol/10^6 cells). Therefore the total ^{3}H label in dTTP was proportional to the overall specific radioactivity of this nucleotide as extracted from the cells. Since the specific radioactivity of this pool continued to rise during the period when the rate of DNA labelling was linear (4–15 min), and did not decrease when the rate of

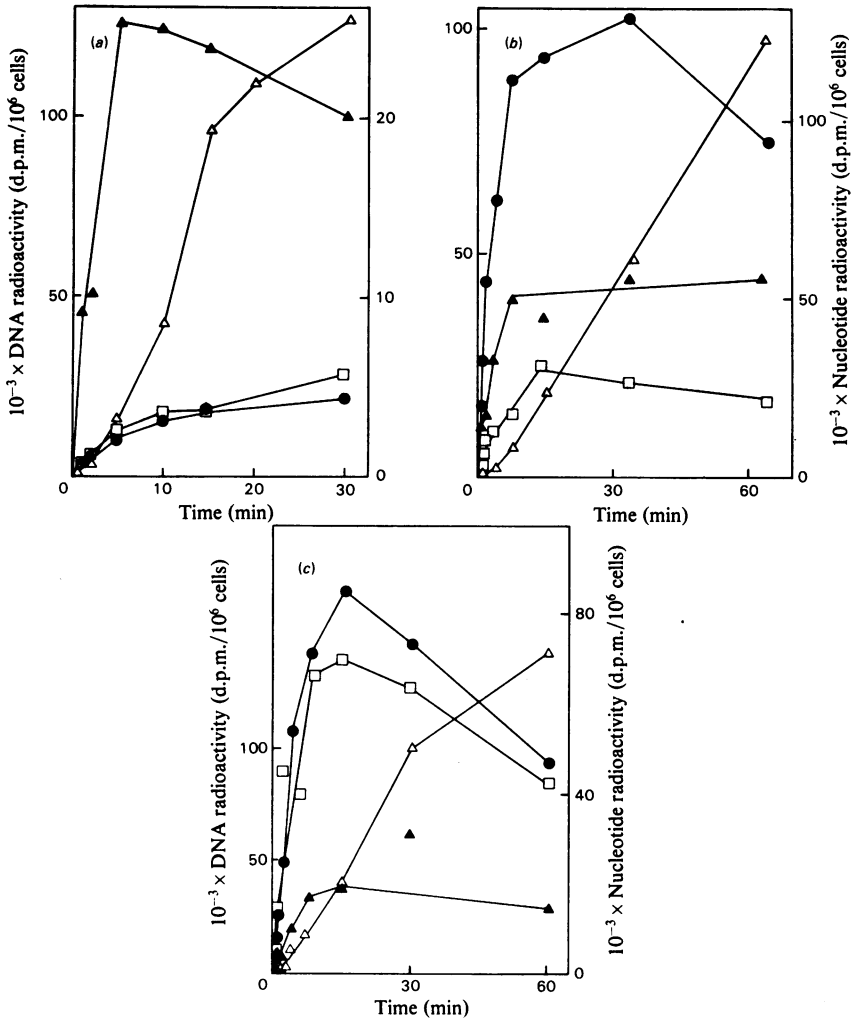

Fig. 1. *Labelling of human cells with [^{3}H]dT at 37°C*

Cells ($5 \times 10^6 \cdot ml^{-1}$) were labelled with [^{3}H]dT ($5\,\mu Ci \cdot ml^{-1}$). Samples (1 ml) were withdrawn at the indicated times and processed for the determination of radioactivity in DNA (△) and in individual nucleotides as described in the Experimental section. ▲, dTMP; □, dTDP; ●, dTTP. (*a*) Phytohaemagglutinin-stimulated lymphocytes; (*b*) bone-marrow cells; (*c*) HPB-ALL cells.

DNA labelling decreased (15–30 min), it appeared that only a part of the intracellular dTTP labelled by exogenous [^{3}H]dT is used as a precursor for DNA synthesis in lymphocytes. However, the appearance and disappearance of label in dTMP paralleled more closely the variation in rate of DNA synthesis with time.

In an attempt to establish why the rate of labelling decreased after 15 min, the following experiment was carried out. A 72 h lymphocyte culture was divided into two and one portion (concentrated to 5×10^6 cells $\cdot$ ml^{-1}) labelled with [^{3}H]dT for 1 h. The cells were collected by centrifugation and processed for the determination of incorporated label. The second portion of lymphocytes was then labelled with the previously used medium. The incorporation of [^{3}H]dT into the second portion of cells was only 17% of that incorporated into the first portion, although less than 0.5% of the [^{3}H]dT had been removed by incorporation into the first portion. We concluded that a proportion of the [^{3}H]dT had been degraded, preventing its incorporation.

Bone-marrow cells labelled with [^{3}H]dT at 37°C

Fig. 1(*b*) shows the results of an experiment in which freshly aspirated bone-marrow cells were labelled with [^{3}H]dT at 37°C. The rate of DNA labelling increased up to about 8 min and then remained constant until at least 60 min. The radioactivity in both the dTTP and dTMP pools also levelled off at 8 min. However, although the label in the dTMP pool remained constant up to 60 min, that in the dTTP pool decreased after 30 min. This experiment did not, therefore, provide unequivocal evidence for compartmentation of dTTP in bone-marrow cells.

HPB-ALL cells labelled with [^{3}H]dT at 22°C

A similar experiment was also carried out using the HPB-ALL cell line. This experiment (Fig. 1*c*) was carried out at 22°C, since DNA-labelling was too rapid to follow at 37°C. The rate of incorporation of [^{3}H]dT into DNA increased up to about 8 min, remained constant between 8 and 30 min and then declined. The label in the dTTP pool (and hence the specific radioactivity of this pool) increased up to about 8 min and then fell. In contrast to the situation in lymphocytes (Fig. 1*a*), the specific radioactivity of the dTTP pool in HPB-ALL cells paralleled the variations in the rate of DNA labelling closely, suggesting that the dTTP in these cells serves exclusively as a precursor for DNA synthesis. The label in the dTMP and dTDP pools also varied in a manner similar to that of the dTTP pool.

Preliminary incubation of cells with [^{3}H]dT at low temperatures followed by 'chase' at 37°C

(*a*) *Phytohaemagglutinin-stimulated lymphocytes.* In order to quantify directly the proportions of thymine nucleotides utilized for DNA synthesis by various human cells, we devised the following protocol. Cells ($5 \times 10^6 \cdot$ ml^{-1}) were preincubated with [^{3}H]dT (73 nM, 5 μCi/ml) at 13°C for 20 min. They were then centrifuged at 500 ***g*** at 13°C for 5 min and resuspended immediately at the same cell concentration in medium prewarmed to 37°C and containing unlabelled 73 nM-dT. Samples were withdrawn at various times during the 'chase' for analysis of label in DNA and nucleotides. Preliminary experiments indicated that, at 13°C, accumulation of label from [^{3}H]dT in intracellular nucleotides was maximal, whereas incorporation into DNA was negligible. Incubation at low temperatures did not affect the capacity of cells to synthesize DNA, since cells held at 13°C for 30 min incorporated [^{3}H]dT on subsequent incubation at 37°C as efficiently as control cells held continuously at 37°C. Furthermore there was no increase in the total measured dTTP pool on incubating cells at 13°C. Therefore events occurring during the 'chase' at 37°C cannot be attributed to a build-up of abnormal concentrations of DNA precursors during the low-temperature incubation.

Fig. 2(*a*) shows the results of an experiment in which lymphocytes were incubated with [^{3}H]dT at 13°C to label the nucleotide pools and then 'chased' at 37°C in unlabelled medium. The results show that, during the centrifugation and 'chase' period, 120000 d.p.m. were lost from the total nucleotide pool, whereas only 30000 d.p.m. were incorporated into DNA, i.e. only 25% of the radioactivity in the total nucleotide pool was available for DNA biosynthesis. Analysis of the radioactivity in individual nucleotide pools showed that 55000 d.p.m. were lost from the dTTP pool during the 'chase'. Therefore a maximum of 55% of the labelled dTTP was available for incorporation into DNA. Incomplete utilization of labelled dTTP was observed in three similar experiments. However, this was not a feature of all lymphocyte cultures (e.g. see Figs. 5 and 6 below). Nevertheless, incomplete incorporation of total labelled thymine nucleotide into DNA was seen in all experiments with normal phytohaemagglutinin-stimulated lymphocytes.

In an attempt to determine the fate of the labelled nucleotide not incorporated into DNA, we solubilized with hyamine hydroxide the protein residue remaining after DNA solubilization of the sample of Fig. 2(*a*). Since this fraction contained negligible label (results not shown) we conclude that none of the label could have been incorporated into protein, e.g. by the transfer of the labelled 5-methyl group of dT into amino acids. Since the acid-soluble fraction, the DNA and protein fractions accounted for all of the intracellular labelled material, the non-incorporated nucleotide must have been lost from the cell,

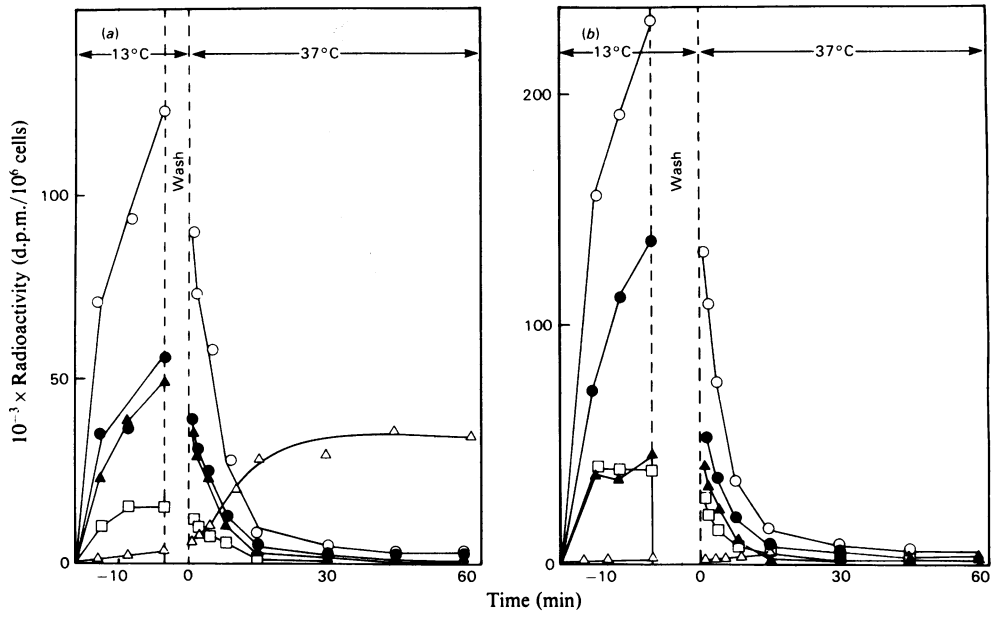

Fig. 2. *Labelling of lymphocytes with [^{3}H]dT at 13°C followed by 'chase' at 37°C*

Phytohaemagglutinin-stimulated lymphocytes (5×10^6 cells·ml^{-1}) were labelled with [^{3}H]dT (5 μCi·ml^{-1}) for 20 min at 13°C. They were collected by centrifugation at 13°C and resuspended at the same concentration in medium prewarmed to 37°C and containing 73 nM unlabelled dT. Samples were withdrawn at the indicated times for determination of radioactivity in DNA (△) and nucleotides (○, ▲, □, ●). ○, Total thymine nucleotides; ▲, dTMP; □, dTDP; ●, dTTP. (*a*) No additions to medium; (*b*) 10 μM-AraC present.

probably by degradation to [^{3}H]dT or some other form that can exit from the cell. Usher & Reiter (1977) have demonstrated the catabolism of dT to β-aminoisobutyric acid in lectin-stimulated lymphocytes. However, this pathway did not appear to be active during S-phase.

The experiment of Fig. 2(*b*) shows that loss of label from nucleotide pools can occur independently of DNA synthesis. This experiment was carried out essentially in the same way as that of Fig. 2(*a*), except that the lymphocytes had been preincubated for 1 h at 37°C with 10 μM-AraC before incubation with [^{3}H]dT at 13°C. AraCTP, the phosphorylated form of AraC, inhibits the replicative DNA polymerase, hence blocking DNA replication (Fridland, 1977). During the 'chase' at 37°C, 230000 d.p.m. were lost from the total nucleotide pool, whereas only 3500 d.p.m. were incorporated into DNA. A rapid loss of label from the pools of dTTP, dTDP and dTMP was evident as in the case of Fig. 2(*a*).

It seemed possible that breakdown of nucleotide pools could be restricted to cells in the population that were not synthesizing DNA, whereas cells in the S-phase utilized their DNA precursors efficiently. Since lymphocytes cannot be synchronized effectively, we approached the question indirectly as follows. Lymphocytes were stimulated with phytohaemagglutinin, and samples were taken at various times and tested for their ability to synthesize DNA (as judged by the incorporation of [^{3}H]dT at 37°C) and to incorporate label from [^{3}H]dT into nucleotide pools at 13°C. Incorporation of [^{3}H]dT into nucleotide pools (at 13°C) or into DNA (at 37°C) did not occur before 24 h of culture. Both processes then increased in concert until the experiment was terminated at 72 h. This suggested that cells not in S-phase did not label their nucleotide pools from exogenous nucleotide, hence they could not have contributed to the non-incorporated nucleotide pools of Fig. 2.

(*b*) *Bone-marrow cells.* Fig. 3(*a*) shows the results of an experiment in which bone-marrow cells were labelled with [^{3}H]dT at 13°C for 20 min and then 'chased' in the presence of unlabelled dT at 37°C. As in lymphocytes, only a fraction (60%) of the total labelled thymine nucleotides were incorporated into DNA during the 'chase'. However, none of the experiments carried out with this tissue showed unequivocally that a part only of the labelled dTTP itself was available for DNA synthesis (cf. Fig. 2*a*). When DNA synthesis was inhibited with AraC (Fig. 3*b*), the nucleotides labelled during incubation with [^{3}H]dT at 13°C were rapidly broken down during subsequent incubation at 37°C.

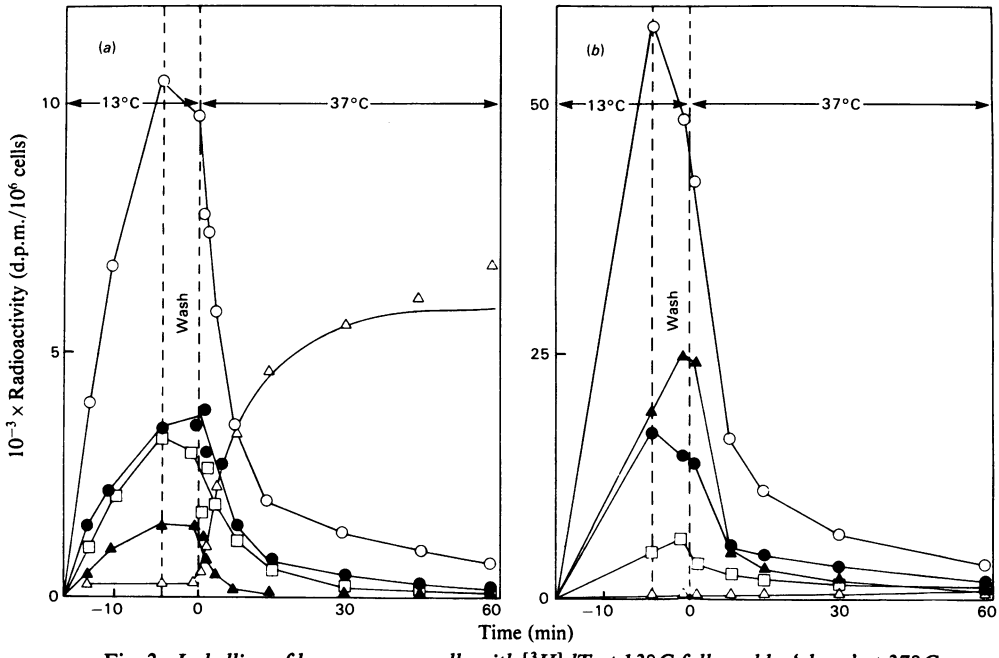

Fig. 3. *Labelling of bone-marrow cells with [^{3}H]dT at 13°C followed by 'chase' at 37°C*
Bone-marrow cells (5×10^6 cells·ml^{-1}) were labelled with [^{3}H]dT (5μCi·ml^{-1}) for 20min at 13°C. They were collected by centrifugation and resuspended at the same concentration in medium prewarmed to 37°C and containing 73nM unlabelled dT. Samples (1ml) were withdrawn at the indicated times for determination of radioactivity in DNA (△) and in nucleotides. ○, Total thymine nucleotides; ▲, dTMP; □, dTDP; ●, dTTP. (*a*) No additions to medium; (*b*) 10μM-AraC present.

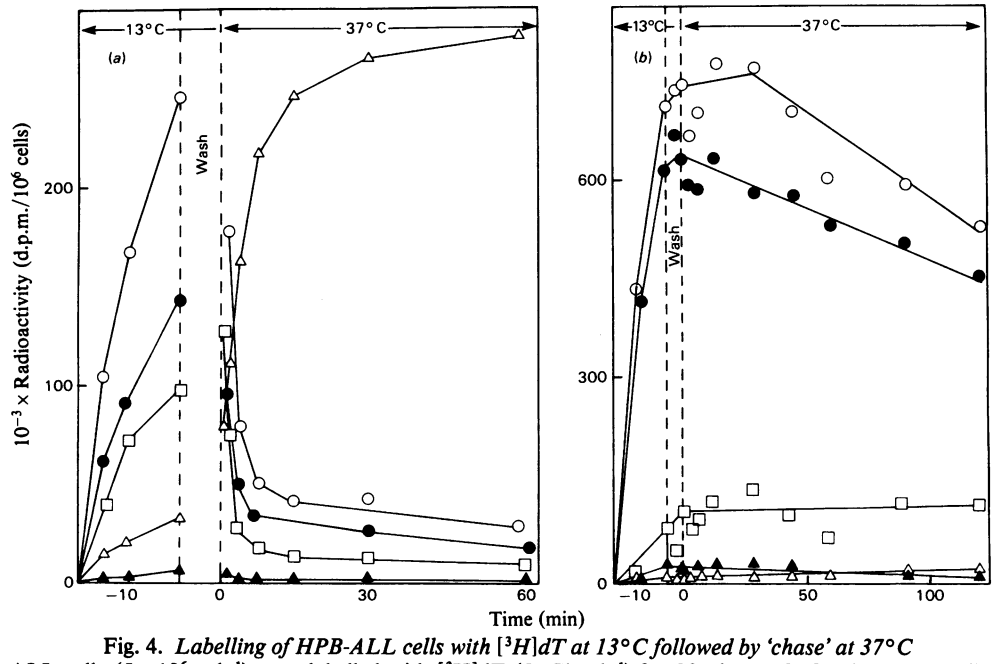

Fig. 4. *Labelling of HPB-ALL cells with [^{3}H]dT at 13°C followed by 'chase' at 37°C*
HPB-ALL cells (5×10^6·ml^{-1}) were labelled with [^{3}H]dT (5μCi·ml^{-1}) for 20min at 13°C. They were collected by centrifugation and resuspended at the same concentration in medium prewarmed to 37°C and containing 73nM unlabelled dT. Samples (1ml) were withdrawn at the indicated times for determination of radioactivity in DNA (△) and in nucleotides (○, ▲, □, ●). ○, Total thymine nucleotides; ▲, dTMP; □, dTDP; ●, dTTP. (*a*) No additions to medium; (*b*) 10μM-AraC present.

(*c*) *HPB-ALL cells.* Fig. 4(*a*) shows the results of an experiment in which HPB-ALL cells were labelled with [^{3}H]dT at 13°C for 20min and subsequently 'chased' in the presence of unlabelled dT at 37°C. In contrast with the results with normal phytohaemagglutinin-stimulated lymphocytes, the decrease of the label in the total pool during the wash and 'chase' (225000d.p.m.) matched almost exactly the incorporation of label into DNA during this period. This experiment, therefore, confirmed our conclusion from the experiment of Fig. 1(*c*) that showed the existence of a single functional dTTP pool in HPB-ALL cells. Fig. 4(*b*) emphasizes the point that little loss of label from thymine nucleotide pools occurred in these cells when DNA synthesis was inhibited in the presence of 10μM-AraC. The label in the thymine nucleotide pools declined only slowly. Of the label present at the beginning of the chase, 90% remained at the end of 1h and 70% at the end of 2h. Surprisingly the label in the dTMP and dTDP pools remained virtually unchanged during the 'chase'. This suggested that the bulk of the cellular dTMP and dTDP are not intermediates in the pathway between dT and dTTP under the conditions of this experiment. Since AraC did not block the flow of label from [^{3}H]dT to [^{3}H]dTTP, we concluded that only a small fraction of the cellular [^{3}H]dTMP and [^{3}H]dTDP served as precursors in [^{3}H]dTTP synthesis in the presence of AraC.

Utilization of labelled nucleotides by lymphocytes from patients with megaloblastic anaemia

Phytohaemagglutinin-stimulated lymphocytes from patients with megaloblastic anaemia display many of the biochemical and morphological characteristics of megaloblasts, the abnormal erythrocyte precursors associated with this syndrome (Das & Hoffbrand, 1970*a*,*b*). Addition of folinic acid and vitamin B_{12} to the culture medium corrects these defects *in vitro*. We therefore exploited this system in order to investigate the utilization of exogenously labelled thymine nucleotide by 'megaloblastic' cells. A 'chase' experiment identical with that described in Fig. 2(*a*) was carried out with untreated (Fig. 5*a*) and vitamin-treated (Fig. 5*b*) lymphocytes from a patient with megaloblastic anaemia due to folate

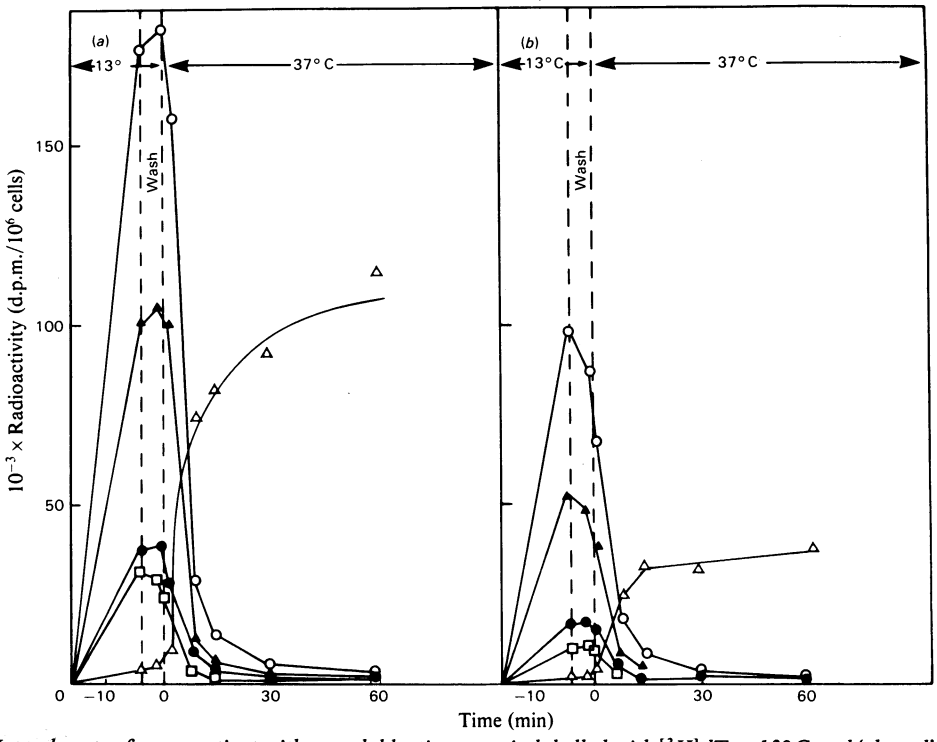

Fig. 5. *Lymphocytes from a patient with megaloblastic anaemia labelled with [^{3}H]dT at 13°C and 'chased' at 37°C*
Phytohaemagglutinin-stimulated lymphocytes from a patient with megaloblastic anaemia (5×10^6cells·ml^{-1}) were labelled with [^{3}H]dT (5μCi·ml^{-1}) for 20min at 13°C. They were collected by centrifugation and resuspended at the same concentration in medium prewarmed to 37°C and containing 73nM unlabelled dT. Samples (1ml) were withdrawn at the indicated times for determination of radioactivity in DNA (△) and in nucleotides (○, ▲, □, ●). ○, Total thymine nucleotides; ▲, dTMP; □, dTDP; ●, dTTP. (*a*) No additions; (*b*) treated *in vitro* with folinic acid (30μg·ml^{-1}) and cyanocobalamin (10μg·ml^{-1}).

deficiency. The uptake of label from [^{3}H]dT into total thymine nucleotides by untreated lymphocytes at 13°C was approx. 2-fold the uptake by vitamin-treated cells. This was probably accounted for by the increased thymidine kinase activity associated with megaloblastic cells (Hooton & Hoffbrand, 1976). Of the labelled thymine nucleotide, 60% was incorporated into DNA by untreated megaloblastic lymphocytes (Fig. 5*a*), whereas only 40% was incorporated in vitamin-treated cells (Fig. 5*b*).

Utilization of labelled nucleotides by lymphocytes treated with methotrexate

Methotrexate inhibits DNA synthesis indirectly by causing a marked decrease in the size of the dTTP pool (Fridland, 1973*b*; Ganeshaguru, 1977). Lymphocytes preincubated for 1 h with 1 μM-methotrexate (Fig. 6*a*) incorporate the same amount of label into its nucleotide pools during a 20 min incubation with [^{3}H]dT as do control lymphocytes (Fig. 6*b*). However, nearly 100% of the labelled thymine nucleotides were incorporated into DNA by the drug-treated cells, whereas only 60% were utilized by the control lymphocytes. Therefore the methotrexate-treated cells utilized available thymidine nucleotides more efficiently than did normal lymphocytes. In order to account for the highly efficient utilization of the total labelled thymine nucleotides by the drug-treated cells, almost all of the ^{3}H-labelled dTMP and dTDP present at the beginning of the 'chase' must have been phosphorylated to dTTP and subsequently incorporated into DNA.

Discussion

The results described here indicate that a functional compartmentation of dNTP pools occurs in phytohaemagglutinin-stimulated human lymphocytes. Labelling of lymphocytes with [^{3}H]dT showed

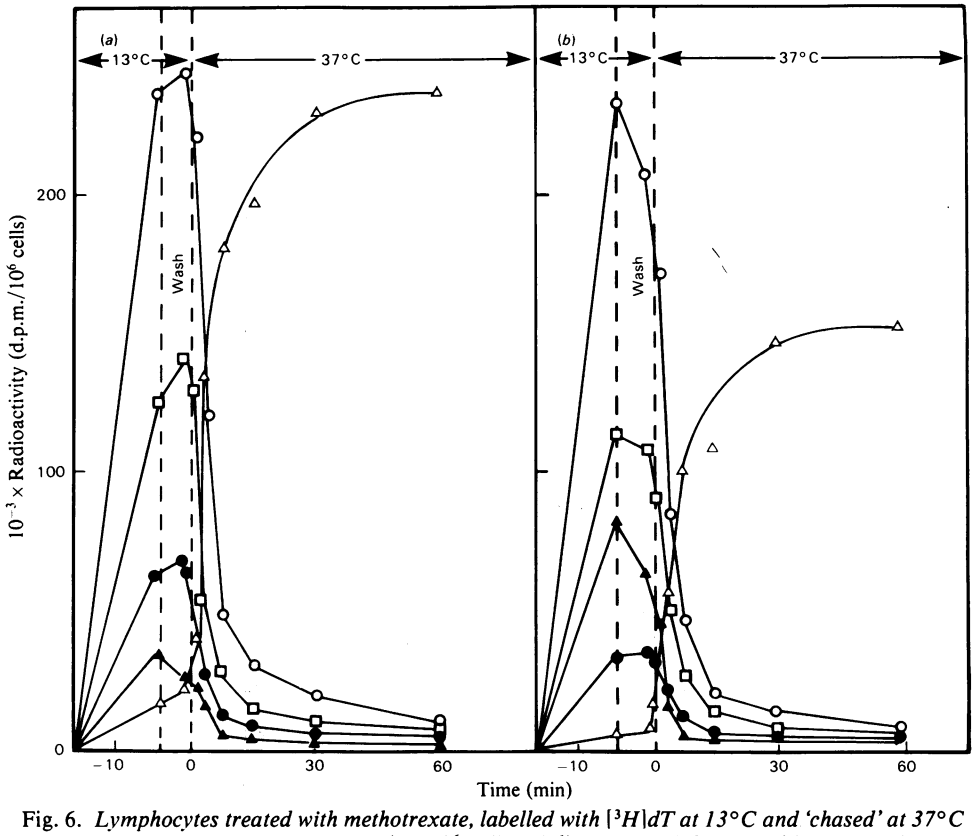

Fig. 6. *Lymphocytes treated with methotrexate, labelled with [^{3}H]dT at 13°C and 'chased' at 37°C*
Phytohaemagglutinin-stimulated lymphocytes (5×10^6 cells·ml^{-1}) pretreated for 1 h with 1 μM-methotrexate were labelled with [^{3}H]dT (5 μCi·ml^{-1}) for 20 min at 13°C. They were collected by centrifugation and resuspended at the same concentration in medium prewarmed to 37°C and containing 73 nM unlabelled dT. Samples (1 ml) were withdrawn at the indicated times for determination of radioactivity in DNA (△) and in nucleotides (○, ▲, □, ●). ○, Total thymine nucleotide; ▲, dTMP; □, dTDP; ●, dTTP. (*a*) 1 μM-Methotrexate present; (*b*) control.

that the specific radioactivity of the dTTP pool did not correlate with the rate of labelling of DNA, suggesting that only a portion of the dTTP labelled from exogenous [^{3}H]dT was available for DNA synthesis. The alternative possibility that dTTP is not the immediate precursor of DNA synthesis (e.g. Werner, 1971*b*) is unlikely given the substrate requirements of a wide range of DNA polymerases from bacterial and eukaryotic sources (Kornberg, 1976). The above conclusion was confirmed in an experiment in which lymphocytes were incubated at 13°C with [^{3}H]dT. Only 25% of the total ^{3}H-labelled nucleotides were incorporated into DNA, the rest being rapidly degraded to a form that was lost from the cells. Chromatographic separation of the labelled nucleotides demonstrated that a maximum of 55% of the [^{3}H]dTTP was available for DNA replication in this experiment. Labelled nucleotides not incorporated into DNA were rapidly degraded, presumably to the nucleoside or some other form that was then lost from the cell. That the loss of label from the nucleotide pools was indeed independent of DNA biosynthesis was confirmed by repeating the above 'chase' experiment in the presence of AraC, a potent inhibitor of DNA synthesis. Lymphocytes not in S-phase did not incorporate [^{3}H]dT into intracellular nucleotide pools. Thus it was unlikely that the rapid loss of label from DNA precursor pools was due to non-S-phase cells in the population.

Since Chinese-hamster ovary cells can be readily synchronized by isoleucine starvation (Tobey & Ley, 1971), we exploited this system (rather than lymphocytes, which are difficult to synchronize) to investigate whether thymine-nucleotide breakdown could be detected in S-phase. In an experiment identical with that shown in Fig. 2(*a*), 49% of the labelled thymine nucleotides were incorporated into the DNA of unsynchronized cells (32% of cells in S-phase), the rest being degraded. In a synchronized S-phase population (more than 99% of cells in S-phase), 45% of labelled nucleotide was incorporated into DNA. Since the proportion of [^{3}H]-thymidine nucleotides that was subject to degradation was very similar in unsynchronized and in synchronized S-phase cells, we concluded that cells not in S-phase did not contribute substantially to the degradation phenomenon.

The conclusion that degradation of labelled thymine nucleotide was a feature of S-phase cells is supported by previous work showing that thymidine kinase activity increased greatly during S-phase in mouse fibroblasts (Adams, 1969*b*). Furthermore the phosphorylation of [^{3}H]dT was confined to S-phase and early G_2-phase in mouse fibroblasts (Adams, 1969*a*,*b*), in the protozoon *Tetrahymena* and in Chinese-hamster fibroblasts (Miller *et al.*, 1964). These workers have also demonstrated the nuclear localization of the pools of thymidine nucleotide derived from [^{3}H]dT. It is therefore unlikely that the nucleotide degradation described in the present study was of an extranuclear pool that was not used for DNA synthesis.

The situation in bone-marrow cells was less clear. Labelling of marrow cells continuously with [^{3}H]dT at 37°C did not provide any clear evidence for compartmentation of any thymine-nucleotide pool. In an experiment in which pools were labelled with [^{3}H]dT at 13°C, only 60% of the total labelled thymine nucleotide was incorporated into DNA during a subsequent 'chase' at 37°C. Once again, this experiment did not demonstrate unequivocally that any single nucleotide pool was compartmentalized. However, when the 'chase' was carried out under conditions in which DNA synthesis was inhibited by AraC, rapid and nearly total breakdown of the labelled nucleotides occurred, confirming that nucleotide breakdown is an alternative metabolic fate for thymine nucleotides in marrow cells.

The established leukaemia cell line HPB-ALL utilized its DNA precursors more efficiently than phytohaemagglutinin-stimulated T-lymphocytes. On labelling Thy-AL leukaemia cells with exogenous [^{3}H]dT, the specific radioactivity of dTTP correlated well with the rate of DNA labelling. The specific radioactivity of dTMP and dTDP also paralleled this pattern. Thus there was no evidence for the compartmentation of any of the thymine-nucleotide pools in this cell line. In complete contrast with the situation in lymphocytes, the labelled nucleotide pools were incorporated almost completely into DNA during a 'chase' after a labelling period at 13°C. Since [^{3}H]dTTP only accounted for 55% of labelled nucleotides at the start of the 'chase', it follows that all of the [^{3}H]dTMP and [^{3}H]dTDP must also have been incorporated into DNA, presumably via phosphorylation to [^{3}H]dTTP. That the labelled nucleotide pools in HPB-ALL cells were not subjected to extensive degradation was confirmed by repeating the 'chase' experiment in the presence of AraC. When DNA replication was inhibited, the label in the nucleotide pools remained elevated up to 2h of 'chase'. Surprisingly, no conversion of [^{3}H]dTMP or [^{3}H]dTDP into [^{3}H]dTTP was detected during the 'chase'. Since [^{3}H]dTTP can be synthesized from [^{3}H]dT in the presence of AraC, we suggest that the bulk of the [^{3}H]dTMP and [^{3}H]dTDP accumulated under the conditions of this experiment were not on the pathway between dT and dTTP. A possible explanation for this observation is that the kinases responsible for the sequential phosphorylation of dT to dTTP are highly localized at restricted sites within the nucleus, possibly near the replication fork. If the [^{3}H]dTMP or [^{3}H]dTDP accumulated during AraC inhibition of HPB-ALL

cells diffused away from the kinases, they would not be further phosphorylated.

Our results suggest that at least two metabolic routes are open to thymine nucleotides in eukaryotic cells. On the one hand they may be incorporated into DNA: on the other, they may enter a pool that does not serve as a precursor for DNA replication. Depending on the cell type, nucleotides in the latter class of pool may be rapidly degraded (e.g. in lymphocytes and bone marrow) or persist for relatively long periods (e.g. in HPB-ALL cells). This is in general agreement with previous results obtained with established human cell lines (Fridland, 1973*a*; Baumunk & Friedman, 1971; Kuebbing & Werner, 1975). These earlier studies, however, differ from the present one in that the authors observed that only a limited proportion of exogenously labelled [^{3}H]dTTP was incorporated into DNA, the rest remaining stable for many hours.

Our results point to a 'functional compartmentation' of thymine nucleotides within the nucleus of S-phase cells, one 'compartment' providing precursors for DNA synthesis distinct from one that does not. The latter compartment may be regulated by degradation in certain cell types (i.e. lymphocytes, bone-marrow cells). One possible way in which such functional compartmentation could be maintained is by the activity of a multienzyme complex that phosphorylates dT to dTTP via a series of enzyme-bound intermediates. Such a mechanism, although totally speculative in eukaryotic cells, operates during the replication of the coliphage T4 (Reddy & Matthews, 1978), maintaining a higher concentration of dNTP species at the fork than would be produced by an unlinked enyzme system. Compartmentation by kinetically coupled enzyme systems has been reviewed by Davis (1972).

The utilization of exogenously labelled thymine nucleotides in lymphocyte DNA synthesis is more efficient when endogenous dTTP biosynthesis is repressed. Untreated lymphocytes from a patient with megaloblastic anaemia incorporated 60% of its labelled nucleotide into DNA, whereas vitamin-treated controls utilized only 40%. When thymidylate biosynthesis was blocked with methotrexate, 100% of the exogenously labelled thymine nucleotide was incorporated into DNA. It thus appears that the enzyme system involved in the biosynthesis and degradation of thymine nucleotides is able to adapt its efficiency in response to the availability of thymine nucleotide.

The present investigation was prompted by the paradoxical observation that the total measured dTTP pool was not decreased in lymphocytes and bone-marrow cells from patients with megaloblastic anaemia (Hoffbrand *et al.*, 1974), although the biosynthesis of this compound is thought to be decreased (Metz *et al.*, 1968; Hoffbrand *et al.*, 1976; Ganeshaguru & Hoffbrand, 1978). Our results here raise the possibility that a pool of nucleotides destined for degradation and unavailable for DNA synthesis could, if sufficiently large, mask changes in the concentration of nucleotides available for replication. Since no information on the size of the degradative pool is available, this view must remain conjectural.

There is considerable interest in the metabolic fates of dNTP species in different lymphoid-cell types in view of the selective toxicity of these compounds towards tumours (Lee *et al.*, 1977). Cell lines derived from lymphocytic leukaemias of thymic origin have been found to accumulate high concentrations of dTTP on exposure to dT, whereas B-lymphoid cell lines did not do so (Fox *et al.*, 1979; Carson *et al.*, 1979). The first of these studies attributed this accumulation to a deficiency of thymidine phosphorylase in T-cell lines, whereas 5′-nucleotidases were implicated in the second study. However, both these studies were carried out by exposing cells to high concentrations of unlabelled dT (60–250 μM). The present work, in which cells were labelled with trace amounts of [^{3}H]dT, confirm the inability of a Thy-AL leukaemia cell line to break down thymidine nucleotides. However, phytohaemagglutinin-stimulated T-lymphocytes and normal bone-marrow cells degraded these compounds very rapidly.

The metabolic fates of dATP and dGTP in different lymphoid cell types are also of interest in view of the selective toxicity of these compounds or their precursors for T-lymphocytes in children born with deficiency of the enzymes adenosine deaminase and purine nucleotide phosphorylase respectively (Giblett *et al.*, 1972; Ochs *et al.*, 1979). Further studies of the possible functional compartmentalization and alternative metabolic fates of dATP and dGTP in T-cells and other lymphoid cells are required.

Note added in proof

A multienzyme complex of DNA-precursor-synthesizing enzymes has recently been demonstrated in Chinese-hamster-embryo fibroblasts (Reddy & Pardee, 1980).

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