Biochemical Pharmacology, Vol. 33, No. 9, pp. 1515–1518, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

DEOXYRIBONUCLEOSIDE TRIPHOSPHATE POOLS IN HUMAN DIPLOID FIBROBLASTS AND THEIR MODULATION BY HYDROXYUREA AND DEOXYNUCLEOSIDES

RONALD D. SNYDER*
Stauffer Chemical Co., Farmington, CT 06032, U.S.A.

(*Received* 5 *August* 1983; *accepted* 25 *October* 1983)

Abstract—Deoxyribonucleoside triphosphate (dNTP) pool levels were examined in synchronized and unsynchronized log phase cultures and in quiescent cultures of human diploid foreskin fibroblasts. dNTP levels were in good agreement with those previously published for human HeLa and lymphoblastic leukemia cells. dCTP and dGTP levels showed only a modest lowering in quiescent as compared to log-phase cells, but dATP and dTTP levels were reduced dramatically in quiescent cultures. Cells synchronized by serum starvation and assayed at the peak DNA synthetic phase (18–21 hr post release) showed substantially higher pools of all four dNTPs. Hydroxyurea treatment reduced only purine dNTPs in both log phase and confluent cells while increasing dTTP and dCTP pools. The effects of deoxynucleosides on dNTP pools were also examined and are discussed in light of current models regarding regulation of purified ribonucleotide reductase formulated from *in vitro* studies.

The consequences of dNTP† pool imbalances have only recently been appreciated. Modulation of the amounts of available dNTPs has been shown to result in decreased fidelity of DNA polymerization leading to mutagenesis, chromosomal breakage phenomena, and toxicity [1]. Deoxyribonucleoside triphosphate (dNTP) pools are closely regulated to within certain ranges by a complex system of positive and negative feedback controls in mammalian cells acting largely via the ribonucleotide reductase. Hydroxyurea (HU) is known to induce alterations in dNTP pools through interaction with the reductase. While the primary effect of HU treatment appears to be a reduction in purine dNTP pools in a variety of mammalian cells [2–5], conflicting reports exist concerning its modulation of pyrimidine dNTP pool levels [4–8]. It seems likely, therefore, that regulation of pool sizes may be under somewhat different controls in different mammalian species. There is no information available regarding dNTP pool sizes in human diploid fibroblasts and little information is available concerning the regulation of ribonucleotide reductase in intact cells [9]. Thus, this study was undertaken to determine normal dNTP pool levels in human fibroblasts under various conditions of growth and in the presence of hydroxyurea or deoxynucleosides.

* Address all correspondence to: Ronald D. Snyder, Ph.D., Stauffer Chemical Co., 400 Farmington Ave., Farmington, CT 06032.

† Abbreviations: dNTP, deoxynucleoside triphosphate; AdR, 2′-deoxyadenosine; GdR, 2′-deoxyguanosine; TdR, thymidine; CdR, 2′-deoxycytidine; HU, hydroxyurea; HPLC, high performance liquid chromatography; PCA, perchloric acid; and Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

MATERIALS AND METHODS

Cell culturing. Human diploid foreskin fibroblasts (HSBP) (obtained from Dr. J. D. Regan, Oak Ridge, TN) were maintained at 37° and 5% carbon dioxide in modified Eagle's medium (MEM) with Hanks' salts supplemented with 10% fetal bovine serum. For all dNTP determinations, the growth medium contained 10% newborn calf serum since HPLC analysis showed undetectable levels of thymidine in this serum. Cells were cultured in plastic T75 tissue culture flasks at an initial seeding density of approximately 1.0×10^5 cells per flask and were used for experiments either while in the exponential log phase, as determined by population doubling indices, or after having been in a completely contact-inhibited quiescent state for 7–10 days, with changes of media every third day. HSBP cells had a doubling time of 22 hr. Log phase cultures contained between 4 and 6% S-phase cells, whereas cultures held in confluency contained less than 0.01% S-phase cells. Synchronized cell cultures were established by allowing initially seeded cells to grow to a density of about 8×10^5 per flask in media containing 10% serum, changing the serum to 0.3% for 7 days to arrest growth, and then raising the serum level to 20% to initiate cycling. It was determined (data not shown) that the peak DNA synthetic period occurred at 18–21 hr after release from low serum arrest. It was at this point that cells were utilized for dNTP pool determinations.

Cells were routinely checked for mycoplasma contamination.

Chemicals. Poly[d(AT)], dAMP, and endonuclease-free *Escherichia coli* DNA polymerase I (at least 8000 units/mg) were purchased from Boehringer-Mannheim. Poly[d(IC)] was purchased from Collaborative Research. dATP, dCTP, dGTP,

Table 1. Deoxyribonucleoside triphosphate pools in normal human diploid fibroblasts*

Culture	Deoxyribonucleoside triphosphate content (pmoles/10^6 cells) dATP	dTTP	dCTP	dGTP
Exponential	23.6 ± 5.4	49.4 ± 11.9	11.4 ± 4.4	6.3 ± 2.1
Quiescent	2.6 ± 0.6	4.4 ± 1.8	9.5 ± 1.6	5.1 ± 0.8
Synchronized in S phase	37.4 ± 7.5	81.6 ± 9.6	20.0 ± 2.7	7.9 ± 1-4

* dNTP pools were determined as described in Materials and Methods. Values are expressed as mean ± S.D.

and TTP were purchased from the Sigma Chemical Co. [8-^{3}H]dATP (22 Ci/mmole), [*methyl*-^{3}H]TTP (30 Ci/mmole), [8-^{3}H]dGTP (12.2 Ci/mmole), [5-^{3}H] dCTP (21 Ci/mmole), and [2-^{3}H]adenosine (28 Ci/mmole) were purchased from Amersham.

Determination of deoxyribonucleoside triphosphate levels in cellular extracts. Determination of dNTPs was made using the procedure of Hunting and Henderson [10] with modifications. Cells in the appropriate cycling stage were either left untreated or were treated with 2 mM HU for 3 hr at 37°. The cells were harvested by trypsinization and scraping. Between 2.0 and 9.0×10^6 cells were pooled in growth medium and collected at 1000 *g* for 6 min at room temperature. The medium was aspirated and the pellets were extracted with 250 μl of 0.4 M PCA for 20 min on ice. The PCA contained [^{3}H]adenosine for determination of dilution. The cellular debris was pelleted and the supernatant fraction (pH < 2) was neutralized by extraction (4–6×) with alamine 336 in Freon TF (resultant pH 5.0 to 5.5). The samples were stored frozen at −20°. An alternative method of extraction was treatment of the cells overnight with 60% methanol. This procedure, though used by many authors, gave artificially elevated pool determinations for dATP (2×), dGTP (4×), dTTP (5×), and dCTP (2×). This effect of 60% methanol has been suggested previously by North *et al.* [11] to result from enzyme contaminants in the methanol extract which are capable of phosphorylating nucleosides. Thus, care should be taken in the choice of extraction procedures. A thorough discussion of this is found in a recent review by Hunting and Henderson [12].

The reaction mixture (160 μl) for the dATP and dTTP assays contained 0.02 O.D.$_{260}$ units poly [d(AT)], 1.8 μmoles $MgCl_2$, 1.8 μmoles dAMP, 18 μmoles HEPES, pH 7.4, and 0.5 Richardson units of DNA polymerase I. The dATP assay also contained 100 pmoles (1 μCi) [^{3}H]dTTP and the dTTP assay also contained 100 pmoles (1.9 μCi) [^{3}H]dATP.

The reaction mixture (160 μl) for the dCTP and dGTP assays contained 0.01 O.D.$_{260}$ units poly [d(IC)], 1.8 μmoles $MgCl_2$, 1.8 μmoles dAMP and 18 μmoles Hepes, pH 7.4. The dCTP assay also contained 240 pmoles (1.9 μCi) [^{3}H]dGTP and 1.0 Richardson unit of DNA polymerase I. The dGTP assay also contained 100 pmoles (2.1 μCi) [^{3}H]dCTP and 1.5 Richardson units of DNA polymerase I.

The reaction was allowed to proceed at 37° for 20 min (dATP and dTTP assays) or 30 min (dCTP and dGTP assays). These times were predetermined to allow for optimal incorporation. Aliquots were removed and counted for incorporated radioactivity as previously described [10].

Standard curves with known amounts of dNTPs were included in each assay. Calculations of pool sizes were made by computer program which corrected for dilution of radioactivity in the assay by dNTPs in the extracts [10].

RESULTS AND DISCUSSION

dNTP pool size determinations were conducted only in low passage human fibroblasts as it has been reported [13] that, with increasing population doublings, the activity of ribonucleotide reductase drops in cultured human fibroblasts. This decrease in reductase activity can manifest itself in lowered dNTP pools in senescent cells (unpublished observations), a probable contributing factor to the observed increased doubling time of those cells.

There is a great deal of variation in dNTP pool levels seen in different experiments with "log-phase" cells. Differences in initial seeding density changed both the length of time to maximum exponential growth and the actual number of cells cycling in that period (data not shown). For this reason, dNTP pool levels shown in Table 1 are represented as the average of between three and five separate determinations. In all experiments with exponentially growing cells, the relative proportions of dNTPs remained fairly constant, with dTTP always comprising the largest pool followed by dATP, dCTP, and dGTP in decreasing order. In cells held at confluency for extended periods of time, there was generally less variation in dNTP pool levels between cultures. As compared to exponential cultures, quiescent cultures were characterized as having only modest decreases in dCTP and dGTP pools but marked decreases in dATP and dTTP pools. The significance of this observation to the regulation of cellular DNA metabolic processes remains to be elucidated. Synchronized cells assayed for dNTP pool content during peak S phase exhibited substantially higher pools of all four dNTPs, as would be expected due to the greater proportion of cycling cells. These pool determinations, however, are still underestimates on a per cell basis since even under the most stringent synchronization procedures only 10–20% of the cells are actually cycling [14].

The pool levels reported here are in general agreement with previous studies on human HeLa cells [15] and leukemic lymphoblasts [16, 17] in that dTTP and

Table 2. Effects of 2mM hydroxyurea treatment on deoxyribonucleoside triphosphate pools in human diploid fibroblasts*

	Deoxyribonucleoside triphosphates (% control)			
	dATP	dTTP	dCTP	dGTP
Exponential cultures	32	120	120	48
Quiescent cultures	40	156	143	10

* dNTP pools were determined as described in Materials and Methods. HU treatment was for 3 hr at 37°. Each number is the average of three determinations.

dATP pools are higher than dCTP and dGTP pools. It is difficult to compare published estimates of pools from one study to another due to inherent differences in the culturing and cycling stages of the cultures and differences in the cell lines employed. It is reassuring, however, that the estimates made in HeLa and leukemic cells fall near to or within the range of pools presented in Table 1 for human diploid fibroblasts. It would thus seem as though the regulation of dNTP pool levels is similar in all of these human cell lines.

The effects of the ribonucleotide reductase inhibitor, hydroxyurea, on dNTP pools are presented in Table 2. It is clear that HU induced a very significant reduction in purine dNTP pools over the 3-hr incubation period while elevating pyrimidine dNTP pools. Doses of HU up to 10 mM were no more effective than 2 mM in eliciting these responses (data not shown). This same differential effect of HU in lowering purine pools has been observed previously in mouse embryo cells [4, 5] and in confluent but not exponentially growing mouse L929 cells [3]. However, dCTP pools have been shown to be decreased by hydroxyurea in phytohemagglutinin-stimulated human lymphocytes [8] and Chinese hamster ovary cells [2, 6]. It has been shown recently [18] that six other inhibitors of ribonucleotide reductase also elevate dTTP pools but, along with their antipurine effect, they also decrease dCTP pool levels. It seems, then, that hydroxyurea may interact with the reductase in a different way from the other inhibitors or that perhaps a separate cytidine diphosphate reductase exists as suggested in rat cells [19, 20] which is differentially sensitive to HU and other inhibitors.

The effects of deoxynucleosides on dNTP pools are presented in Table 3. It is clear that treatment of cells with any of the four deoxynucleosides leads to an elevation of the corresponding triphosphate. It is interesting that log phase and confluent cultures respond to different degrees in this regard. dATP pools were 100-fold higher in log cells than in confluent cells following treatment with AdR. Likewise, log phase cells produced significantly more (15-fold) TTP than confluent cells. On the other hand, dGTP was formed in roughly equal amounts under both conditions and dCTP was formed to a greater extent in confluent cells. This complex pattern most certainly reflects not only the levels of the respective kinases but also the levels of degradative enzymes (e.g. deaminases).

The effects on dNTP pools generally agree with the current model for regulation of ribonucleotide reductase [9] formulated largely from *in vitro* studies, and with recent reports on regulation in intact human cells [21, 22]. AdR treatment led to elevated dATP pools which allosterically inhibited the reduction of CDP and GDP. The fact that TTP pools were not affected significantly probably reflects slower turnover rather than an effect at the level of UDP reduction since 500 μM AdR inhibited DNA synthesis by 90% (data not shown). We cannot completely rule out the possibility, however, that TdR salvaged from the serum contributed to the observed TTP pools. Elevated TTP pools following TdR treatment activated reduction of GDP, and the subsequently formed dGTP activated reduction of ADP to dATP. This elevated dATP then depressed CDP reduction. GdR elevated dGTP which led again to an elevation in dATP and a depression in dCTP levels. There was little, if any, effect on TTP pools. dCTP is not generally regarded as an effector of ribonucleotide

Table 3. Effects of deoxynucleosides on cellular dNTP pools*

Treatment	Culture	Deoxynucleoside triphosphate content (pmoles/10^6 cells) dATP	dTTP	dCTP	dGTP
500 μM AdR	Log	623 (2640)	57 (115)	6 (51)	1 (15)
	Confluent	6 (230)	4 (94)	5 (57)	3 (56)
500 μM GdR	Log	44 (185)	59 (119)	4 (32)	57 (900)
	Confluent	5 (196)	5 (110)	7 (75)	57 (1116)
500 μM TdR	Log	42 (178)	232 (470)	5 (40)	10 (154)
	Confluent	3 (110)	15 (332)	7 (70)	8 (150)
500 μM CdR	Log	22 (93)	90 (183)	20 (176)	12 (183)
	Confluent	3 (134)	5 (108)	66 (700)	7 (134)

* All figures are the average of three to five determinations from different extracts. Numbers in parentheses are percent control levels. All determinations were made at 3 hr.

reductase; thus, some of the results presented here are curious. Elevation of dGTP pools by CdR has been observed previously with human lymphoblast (Molt-4F) reductase [23], consistent with our results but inconsistent with studies on intact human leukemic cells in which no such effect was demonstrated [21]. It is likely that the elevated dGTP pools occurred as a consequence of increased activation of GDP reduction by TTP that was produced in enhanced amounts in CdR-treated cells possibly via dCMP deaminase. Log phase and quiescent cells behaved qualitatively very similarly in response to AdR, GdR, and TdR treatment, the magnitude of the effect depending on the degree of elevation of the respective triphosphate. The apparent differences in response of cells under different cycling conditions to CdR treatment are not as clear and tend to point up the problems involved in dissection of regulatory pathways in intact cell systems.

This study provides the first observations concerning dNTP pools in cultured human diploid fibroblasts and their regulation at the level of the reductase. It appears that the regulation is very similar to that seen both in the *in vitro* systems and in intact mammalian cell systems. Moreover, these effects of HU on dNTP levels in human fibroblasts are consistent with previously published data derived from studies on purified reductase and from other human cells.

REFERENCES

1. B. A. Kunz, *Environ. Mutagen.* **4**, 695 (1982).
2. L. Skoog and G. Bjursell, *J. biol. Chem.* **249**, 6434 (1974).
3. R. L. Adams, S. Berryman and A. Thomson, *Biochim. biophys. Acta* **240**, 455 (1971).
4. P. Reichard, *Adv. Enzyme Regulat.* **10**, 3 (1972).
5. L. Skoog and B. Nordenskjold, *Eur. J. Biochem.* **19**, 81 (1971).
6. J. Clarkson, *Mutation Res.* **529**, 273 (1978).
7. F. W. Scott and D. R. Forsdyke, *Biochem. J.* **190**, 721 (1980).
8. G. Tyrsted, *Biochem. Pharmac.* **31**, 3107 (1982).
9. D. Hunting and J. F. Henderson, *CRC Crit. Rev. Biochem.* **13**, 325 (1982).
10. D. Hunting and J. F. Henderson, *Can. J. Biochem.* **59**, 723 (1981).
11. T. W. North, R. K. Bestwick and C. K. Mathews, *J. biol. Chem.* **255**, 6640 (1980).
12. D. Hunting and J. F. Henderson, *Meth. Cancer Res.* **20**, 245 (1982).
13. J. E. Dick and J. A. Wright, *Mech. Ageing Dev.* **20**, 103 (1982).
14. M. R. Duncan, M. J. Robinson and R. T. Dell'Orco, *Cell Biol. Int. Rep.* **6**, 369 (1982).
15. J. Horden and J. F. Henderson, *Can. J. Biochem.* **60**, 422 (1981).
16. R. F. Kefford and R. M. Fox, *Cancer Res.* **42**, 324 (1982).
17. G. B. Grindey, M. C. Wang and J. J. Kinahan, *Molec. Pharmac.* **16**, 601 (1979).
18. R. D. Snyder, *Genetic Consequences of Nucleotide Pool Imbalances*, Plenum Press, New York, in press.
19. T. Collins, F. David and J. L. Van Landsder, *Fedn Proc.* **31**, 641 (1972).
20. E. Baril, B. Baril and H. Elford, *Proc. Am. Ass. Cancer Res.* **13**, 334 (1972).
21. D. D. Ross, S. A. Akman, A. W. Schreder and N. R. Bachur, *Cancer Res.* **41**, 4493 (1981).
22. M. H. N. Tattersall, K. Ganeshaguru and A. V. Hoffbrand, *Biochem. Pharmac.* **29**, 1495 (1975).
23. C-H. Chang and Y-C. Cheng, *Cancer Res.* **39**, 5087 (1979).