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RELATIONSHIP BETWEEN RIBO- AND DEOXYRIBONUCLEOTIDE CONCENTRATIONS AND BIOLOGICAL PARAMETERS IN CULTURED CHINESE HAMSTER OVARY CELLS*

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Abstract—The relationship between ribo- and deoxyribonucleotide concentrations, growth rate and cell viability has been studied. Nucleotide pools were manipulated using mycophenolic acid, pyrazofurin, phosphonoacetyl-L-aspartate (PALA) or thymidine. Growth rate, cell viability, nigrosin exclusion, progression through the cell cycle, and the rates of nucleic acid synthesis were measured. The observed qualitative relationships between nucleotide concentrations and growth rate can be rationalized in terms of the effects of changes in substrate availability on nucleic acid synthesis. However, there is no discernible relationship between changes in nucleotide concentrations and the loss of cell viability. More detailed studies will be required to elucidate each step in the complex process leading from the initial changes in nucleotide concentrations to cell death.

Inhibition of cell growth and loss of cell viability are associated with altered intracellular nucleotide concentrations following treatment with a number of purine and pyrimidine antimetabolites, some naturally occurring purines and pyrimidines, and other agents that affect purine and pyrimidine metabolism. This qualitative relationship between nucleotide concentrations and important biological parameters has been extended, in some cases to a quantitative or semi-quantitative relationship between changes in concentrations of one or another nucleotide and inhibition of growth (e.g. Refs. 1–4); however, only in recent years have concentrations of all the purine and pyrimidine ribo- and deoxyribonucleotides been regularly measured in such studies. In contrast to studies that have measured growth inhibition, few if any detailed investigations have been made of the relationship(s) between nucleotide concentrations and loss of cell viability.

In an attempt to study the quantitative relationships among nucleotide concentrations, cell growth and cell viability, we have measured growth rate, cloning efficiency, nigrosin exclusion, progression through the cell cycle, and the specific-activity corrected rates of DNA and RNA syntheses in cultured Chinese hamster ovary cells in which nucleotide concentrations were manipulated in several different ways.

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MATERIALS AND METHODS

Materials. [6-^{3}H]Uridine, 22.4 Ci/mmole, and [8-^{3}H]guanine, 15 Ci/mmole, were obtained from the New England Nuclear Corp., Boston, MA; and [5-^{3}H]cytidine, 20 Ci/mmole, [5-^{3}H]deoxycytidine, 20 Ci/mmole, [methyl-^{3}H]thymidine, 50 Ci/mmole, and [2-^{3}H]hypoxanthine, 15 Ci/mmole, from Moravek Biochemicals, Los Angeles, CA; [8-^{3}H]guanosine, 5.7 Ci/mmole, was obtained from the Schwarz-Mann Co., Orangeburg, NY. [^{3}H]dATP, 11 Ci/mmole, [^{3}H]dTTP, 14 Ci/mmole, [^{3}H]dCTP, 16.8 Ci/mmole, and [^{3}H]dGTP, 15 Ci/mmole, were obtained from ICN Pharmaceuticals Inc., Irvine, CA.

DNA polymerase I from *Escherichia coli* was obtained from Boehringer-Mannheim Corp., DNase I, snake venom phosphodiesterase and alkaline phosphatase were obtained from the Sigma Chemical Co., St. Louis, MO. Poly[d(IC)] was purchased from Miles Laboratories, Elkhart, IN, and poly[d(AT)], non-radioactive purine and pyrimidine bases, nucleosides and nucleotides, from the Sigma Chemical Co.

Mycophenolic acid was a gift of Dr. T. J. Franklin. PALA (phosphonoacetyl-L-aspartate) and pyrazofurin were obtained from the Division of Cancer Treatment, U.S. National Cancer Institute, Bethesda, MD.

Cell culture. Chinese hamster ovary-Kl cells, obtained from Dr. G. Whitmore (Ontario Cancer Institute, Toronto, Canada), were grown in alpha-MEM medium containing 10% dialyzed fetal calf serum (Grand Island Biological Co., Grand Island, NY). The cells were grown in 125 ml bottles on a model G-2 Gyrotatory shaker (New Brunswick Scientific Co., New Brunswick, NJ) at 200 rpm. The

average doubling time was 12 hr and growth was exponential to 0.8–1.0 × 10^6 cells/ml. The cells were routinely tested for mycoplasma and found to be negative.

Biological parameters. Cell density and the population volume distribution were determined using a model Z_F Coulter Counter equipped with a 100-channel Coulter Channelyzer II. The cell cycle distribution was determined by flow microfluorometry using a Bio-Physics model 4800A Cytofluorograph equipped with a 100-channel model 2100 pulse height analyzer. Cells were collected in a clinical centrifuge, resuspended to 400,000 cells/ml in 0.05 mg/ml propidium iodide in 0.1% sodium citrate, and stained for 20–40 min on ice. In any given experiment, the staining time was identical for all the samples.

Cell viability was determined using a simple colony forming assay as follows. Cell density was measured, followed by three serial dilutions using warm, gassed medium, to give a final cell density of 50–100 cells/ml for untreated cells. When necessary, higher final densities were used for drug-treated cells to allow accurate determination of cell kill. Cell suspension (2 ml) was added to each of three warmed, gassed 25 cm^2 plastic tissue culture flasks. After incubation for 7 days at 37° in a humidified atmosphere of 5% CO_2 in air, the medium was poured off, and the colonies were stained with 0.1% crystal violet in physiological saline for *ca.* 30 min and then counted. Control cloning efficiencies were routinely greater than 90%.

Cell integrity at the time of cloning was determined for each drug treatment conditions by measuring nigrosin exclusion.

Cell extraction. Preparation of extracts for nucleotide pool size measurements was as follows: 0.25 to 4.0 × 10^7 cells were centrifuged at 1000 *g* for 2 min at 4°. The medium was aspirated and the tube was recentrifuged at 1000 *g* for 5 sec to remove medium from the centrifuge tube wall. The pellet was extracted on ice with 0.4 M PCA* containing [^{3}H]adenosine for determination of dilution. After 30 min the extract was centrifuged and the supernatant fraction was removed and neutralized by extraction with 0.5 M Alamine 336 (tricapryl tertiary amine) in Freon-TF (trichlorotrifluoroethane) [5]. Supernatant fractions were stored at −20°. HPLC analysis of nucleotides in samples stored for several weeks showed no nucleotide breakdown.

Nucleotide concentrations. Ribonucleotide concentrations were measured by HPLC using a modified Varian Aerograph 1000 liquid chromatograph equipped with a Spectra-Physics model 740P pump, a Waters Associates U6K injector, and a Spectra-Physics Autolab Minigrator. A Partisil 10 SAX anion exchange column (Whatman) was used, and the nucleotides were routinely eluted isocratically at 38° with 0.25 M KH_2PO_4, 0.5 M KCl, pH 4.5, at 1.3 ml/min; this allowed quantitation of ADP and the triphosphates. Detection was at 254 nm and at 0.02 absorbance units full scale. The peaks were automatically integrated and also checked by planimetry. The absolute amounts of nucleotides in the samples were calculated on the basis of peak areas of nucleotide standards which were chromatographed frequently. Accuracy and reproducibility (S.D., three determinations) of all four ribonucleoside triphosphate measurements were 94% and 4% respectively.

Deoxyribonucleoside triphosphate concentrations were measured using a modification of the DNA polymerase procedure [6]. Accuracy and reproducibility, respectively, were as follows: 98 and 2.5% for dATP, 96 and 5.8% for dTTP, 93 and 4.3% for dCTP, and 78 and 8.5% for dGTP. The following controls were performed. Background incorporation (i.e. in the absence of the limiting nonradioactive deoxyribonucleotide) was always measured. In addition, standards were added to cell extracts to determine if the assay was affected by the extracts, and checks were made to demonstrate that the assays were independent of the amount of extract used. Finally, time courses were always performed both with standards and with each cell extract to ensure that the maximum incorporation was reached at the same time under all conditions.

Rates of RNA and DNA synthesis. Determination of the true net rate of DNA or RNA synthesis requires that the results of simple precursor incorporation data be corrected for dilution of the radioactivity by the intracellular nucleotide pools; it is particularly important for such corrections to be made when the nucleotide concentrations of cells are varied, as in the present work. This "specific-activity-corrected isotope incorporation" method involves determination of the steady-state specific activity of the immediate precursor of DNA or RNA by measuring both the concentration of the ribo- and deoxyribonucleoside triphosphates and the total radioactivity in each pool. These measurements are made under steady-state conditions, which is defined as the period when the rate of incorporation of radioactivity into DNA and RNA is linear and, therefore, when the immediate precursors have reached their maximum specific activities. The true net rate of DNA or RNA synthesis, in pmoles per min, is calculated by dividing the steady-state rate of incorporation into DNA or RNA, in counts/min, by the specific activity of the immediate precursor pool, in counts/min per pmole. In preparation for measuring the rates of DNA and RNA synthesis, time courses for up to 90 min were performed to determine the approximate time required for the incorporation of each radioactive precursor into nucleic acids to become linear and also to ensure that the incorporation rate was not being affected by either precursor depletion or radiation effects. Under experimental conditions the rate of incorporation of radioactive precursor into DNA or RNA was calculated from the slope of the incorporation curve which was linear from at least 30 to 60 min. The specific activity of the immediate precursor pool was measured at 30 and 60 min.

CHO-Kl cells at a density of 200,000–400,000 cells/ml were labelled with radioactive precursors at concentrations between 0.20 and 1.8 μM and at specific activities between 5.5 and 50 Ci/

* Abbreviations: PCA, perchloric acid; HPLC, high performance liquid chromatography; PPO, 2,5-diphenyloxazole; and POPOP, 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]benzene.

mmole. Portions of 1 or 5 ml were removed over a 90-min time course. The cells were pelleted by centrifugation and extracted with 50 μl of ice-cold 0.4 M PCA containing [^{14}C]adenine for determination of dilution. After 30 min on ice, the samples were centrifuged. The supernatant fraction was removed and neutralized with an equal volume of 0.5 M Alamine-336 in Freon-TF [5]. Ten microliters was counted in Triton X-100 counting fluid (0.4% PPO, 0.02% POPOP, in 1 part Triton X-100 and 2 parts toluene) to measure the [^{14}C]adenine radioactivity which was used to calculate the dilution occurring during extraction. The tritium in the extract represented mainly label incorporated into nucleotides as well as some label from the original precursor. The neutralized extracts were stored at −20°.

The acid-insoluble pellet, containing labelled DNA and RNA, was washed three times with 0.5 ml of ice-cold 0.4 M PCA, and then dissolved in 200 μl of 0.2 N KOH and incubated at 37° for 18 hr to hydrolyze the RNA. The DNA was precipitated on ice by addition of 4 M PCA to a final concentration of 0.3 M. After centrifugation, the supernatant fraction containing the 2′- and 3′-monophosphates from the hydrolyzed RNA was removed and neutralized with an equal volume of Alamine-Freon. Twenty-five microliters was counted in Triton X-100 counting fluid before chromatography to determine the total radioactivity in the RNA nucleotides. The DNA pellet was washed three times with ice-cold 0.4 M PCA and then dissolved in 50 μl of 100 mM Tris buffer, pH 8.0. $MgCl_2$, NaCl, and DNase I were added to final concentrations of 2.5 mM, 5.0 mM and 50 μg/ml, respectively, and the solution was incubated for *ca.* 18 hr at 37°. Ammonium acetate, $MgCl_2$, snake venom phosphodiesterase, and alkaline phosphatase were added to final concentrations of 100 mM, 2 mM, 50 μg/ml (1×10^{-3} U), and 165 μg/ml (182 U), respectively, and the solution was incubated for 37° for *ca.* 18 hr. If alkaline phosphatase was not used, conditions which resulted in complete DNA hydrolysis produced both deoxyribonucleoside-monophosphates and deoxyribonucleosides, complicating the separation procedure. Twenty-five microliters of the DNA hydrolysate was counted in Triton X-100 counting fluid to determine the total radioactivity before chromatography.

The acid soluble purine and pyrimidine ribo- and deoxyribonucleotides were separated by two-dimensional chromatography on PEI-cellulose thin-layer plates using a modification of the method of Crabtree and Henderson [7]. The plates were prepared by attaching a wick and washing overnight with 1.8% ammonium formate, 2% boric acid, pH 7.0, followed by an overnight wash with 50% methanol in water. After drying, the plates were stored at 4° over a desiccant. Markers and 10 or 20 μl of the extract were spotted, and the plates were run overnight in 50% methanol in water to wash bases and nucleosides onto the wick. The wicks were removed, the plates were dried, and fresh wicks were attached. The plates were developed to 8 cm above the origin with 1.8 M ammonium formate, 2% boric acid, pH 7.0, followed (immediately) by development in 3.3 M ammonium formate, 4.2% boric acid, pH 7.0, until the leading marker was near the wick. The wick was removed and the plates were immersed in methanol for 15 min. After drying, the plates were developed in the second dimension to 2.5 cm above the origin with 0.5 M sodium formate, pH 3.4, then to 8 cm above the origin in 2.0 M sodium formate, pH 3.4, and finally up to the wick or until the triphosphates were well separated, in 4.0 M sodium formate, pH 3.4. At times, the leading monophosphates were run onto the wicks to achieve a better separation. After drying and removal of the wicks, the plates were dipped in methanol to remove the salt in preparation for oxidation. The spots were visualized with u.v. light and scraped onto Whatman No. 1 paper and oxidized, in a Packard 305 oxidizer, to [^{3}H]H_2O. The counting efficiency was 30–34% and the recovery at the oxidation step ranged from 90 to 95%. The effects of carryover of [^{3}H]H_2O from one sample to another were minimized both by adjusting the order in which the samples were burned and by the frequent oxidation of blanks.

RESULTS

To study relationships between changes in nucleotide concentrations and biological parameters, it was necessary first to establish conditions under which both nucleotide concentrations and growth inhibition varied over a wide range. Nucleotide pools were, therefore, manipulated using mycophenolic acid, an inhibitor of inosinate dehydrogenase (EC 1.2.1.14) [8, 9]; pyrazofurin, an inhibitor of orotidylate decarboxylase (EC 4.1.1.23) [10]; PALA, an inhibitor of aspartate transcarbamylase (EC 2.1.3.2) [11]; and thymidine.

Table 1 shows the amount of growth inhibition

Table 1. Effects of several drug treatment conditions on growth rate and viability of CHO cells*

Treatment condition	Growth rate (% of control)	Cloning efficiency (% of control)
0.1 μM Mycophenolic acid; 25 hr	100	100
0.5 μM Mycophenolic acid; 25 hr	67	78
1.0 μM Mycophenolic acid; 24 hr	21	32
2.0 μM Mycophenolic acid; 22 hr	17	4
10.0 μM Mycophenolic acid; 25 hr	0	0
0.01 μM Pyrazofurin; 24 hr	83	95
0.04 μM Pyrazofurin; 20 hr	71	ND
0.1 μM Pyrazofurin; 20 hr	56	ND
0.2 μM Pyrazofurin; 22 hr	30	49
2.0 μM Pyrazofurin; 24 hr	35	25
100 μM PALA; 24 hr	84	89
500 μM PALA; 22 hr	40	41
100 μM Thymidine; 24 hr	100	ND
500 μM Thymidine; 24 hr	79	ND
2.0 mM Thymidine; 24 hr	63	86

* Values reported for growth rate are representative of results of two to four experiments. Values for cloning efficiency are means of duplicate measurements in two separate experiments. For all treatment conditions more than 90% of the cells excluded dye at the time of cloning. Growth rates are expressed as the average growth rate from the time of addition of drug. The values for untreated cells for growth rate and cloning efficiency were 1 doubling per 12 hr and 90% respectively. ND = not determined.

Table 2. Nucleotide pools, growth rate, and viability during treatment of CHO cells with 2.0 μM mycophenolic acid*

Treatment time (hr)	ATP	GTP	CTP	UTP	dATP	dGTP	dCTP	dTTP	Growth rate	Cloning efficiency
	(% of control)									
2	96	30	94	119	82	21	66	137	ND	106
4	81	17	123	142	111	15	91	179	ND	86
6	88	18	118	146	163	18	101	214	58	66
22	124	29	135	139	57	31	126	300	0	4

* Nucleotide values for untreated cells, in pmoles/10^6 cells, were: ATP, 5580; GTP, 1070; CTP, 1230; UTP, 2390; dATP, 39; dGTP, 12; dCTP, 223; and dTTP, 58. ND = not determined.

and loss of cell viability caused by these agents at several concentrations. At the time of cloning, nigrosin exclusion was also measured and it was found that more than 90% of the cells excluded dye under all conditions; this demonstrates the integrity of the membranes of the cells being cloned. As well, the debris peak on the Coulter Channelyzer was approximately the same size in both treated and control cells, indicating that cell lysis was not occurring. Finally, the ratio of ATP to ADP was determined by HPLC on cell extracts prepared at the time of cloning; it was approximately the same for both control and treated cells (> 10:1), thus indicating that the energy generating and utilizing reactions of the cells were in their normal balance. The treatment conditions chosen for more detailed study were: 2 μM mycophenolic acid, 0.2 μM pyrazofurin, 500 μM PALA, and 2.0 mM thymidine.

Mycophenolic acid. Table 2 compares nucleotide pool sizes, growth rate, and cell viability during treatment of CHO cells with 2.0 μM mycophenolic acid. Changes occurred in all the nucleoside triphosphate pools and, as expected, those of GTP and dGTP decreased to the greatest extent; the largest increases were in dTTP and in dATP. The GTP and dGTP pool sizes changed very little after the first 2 hr of treatment, whereas the dTTP pool continued to increase after 6 hr of treatment. A substantial change also occurred in the dATP pool between 6 and 22 hr of treatment with a decrease from 163% to 57% of control. Cell viability was not affected after 2 hr of treatment, only decreasing to 86% of control after 4 hr.

Table 3. Rates of DNA and RNA syntheses after treatment of CHO cells with 2.0 μM mycophenolic acid for 2 hr*

Precursor	Rate of DNA synthesis (% of control)	Rate of RNA synthesis (% of control)
[^{3}H]Deoxycytidine	21	
[^{3}H]Thymidine	17	
[^{3}H]Hypoxanthine	22	23
[^{3}H]Guanine		27
[^{3}H]Uridine		30
Average	20	27

* Results are averages of duplicate measurements in two separate experiments.

The only pool size change which correlated well with the decrease in viability was the increase in dTTP. However, it is unlikely that the loss of viability was a result of the increase in dTTP alone because when the dTTP pool was elevated to 400–500% of control using 100μM thymidine this condition was not growth inhibitory.

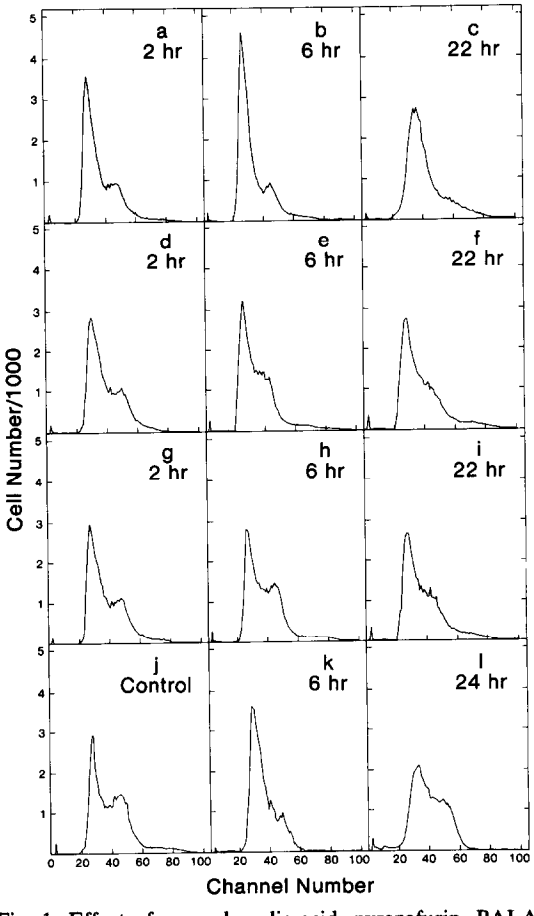

Fig. 1. Effect of mycophenolic acid, pyrazofurin, PALA and thymidine on the cell cycle distribution of CHO cells. Cells were treated for 2, 6 and 22 hr with 2 μM mycophenolic acid (a, b and c), or 0.2 μM pyrazofurin (d, e and f), or 500 μM PALA (g, h and i), or for 6 and 24 hr with 2.0 mM thymidine (k and l). A control cell cycle distribution was determined at each time point and a representative distribution is shown (j). In each case 50,000 cells were counted.

Table 4. Nucleotide pools, growth rate, and viability during treatment of CHO cells with 0.2 μM pyrazofurin*

Treatment time (hr)	ATP	GTP	CTP	UTP	dATP	dGTP	dCTP	dTTP	Growth rate	Cloning efficiency
	(% of control)									
2	119	128	16	25	112	50	31	81	ND	91
4	111	109	2	7	109	42	13	74	ND	78
6	108	106	3	7	113	43	13	83	106	78
22	134	127	9	10	53	46	11	68	17	41

* Control nucleotide concentrations are reported in Table 2. ND = not determined.

The extent to which the substantial decreases in the concentrations of GTP and dGTP (alone or in combination with other nucleotide changes) would inhibit the synthesis of nucleic acids was considered next. The results shown in Table 3 demonstrate that the synthesis of both DNA and RNA were inhibited to similar extents.

To determine the effect of the nucleotide pool size changes caused by mycophenolic acid on the progression of cells though the cell cycle, the cell cycle distribution of the population was measured. Figure 1 (a, b and c) shows the gradual accumulation of cells near the G_1/S border and the disappearance of cells from the late S and G_2 phases of the cell cycle. This process was barely discernible after 2 hr of treatment but was evident after 6 hr of treatment.

No change in modal cell volume of the population had occurred by 6 hr of treatment; however, by 22 hr the volume had increased to 167% of control.

Pyrazofurin. Table 4 compares nucleotide pool sizes with cell growth rate and cell viability during treatment with 0.2 μM pyrazofurin. Changes occurred in all the nucleoside triphosphate pools with the largest changes occurring in the CTP, UTP and dCTP pools. The changes in these pools were small after the first 2 hr of treatment. As observed with mycophenolic acid treatment, the dATP pool decreased between 6 and 22 hr of treatment. The growth rate was unaffected at 6 hr of treatment, but the cloning efficiency had decreased to 78% of control. Between 6 and 22 hr both the growth rate and viability decreased substantially, and the largest nucleotide pool size change was the decrease in dATP. None of the changes in the nucleotide pools correlated well with the loss of viability over the period studied.

Table 5. Rates of DNA and RNA syntheses after treatment of CHO cells with 0.2 μM pyrazofurin for 2 hr*

Precursor	Rate of DNA synthesis	Rate of RNA synthesis
	(% of control)	
[^{3}H]Thymidine	48	
[^{3}H]Deoxycytidine	30	
[^{3}H]Adenine	42	55
[^{3}H]Guanosine		44
[^{3}H]Uridine		52
Average	40	50

* Results are averages of duplicate measurements in two separate experiments.

The results shown in Table 5 demonstrate that the decreases in nucleotide concentrations resulted in inhibition of both DNA and RNA syntheses.

Flow microfluorometry (Fig. 1, d, e and f) showed only slight changes in the cell cycle distribution during the first 6 hr of treatment, with a small decrease in the proportion of cells in late S and G_2 and a corresponding increase in cells in G_1 or early S phase. Since there was no growth inhibition during the initial 6 hr of treatment, the cells must have been dividing at control rates but not progressing as fast through S phase. There was little or no change in the distribution between 6 and 22 hr. Therefore, unlike mycophenolic acid, pyrazofurin did not cause substantial synchronization of the cells, probably because cell growth was not inhibited completely.

No changes in modal cell volume had occurred after 6 hr of treatment and by 22 hr the modal volume had only increased to 125% of control.

PALA. Table 6 compares nucleotide pool sizes with growth rate and cell viability during treatment with 500 μM PALA. As with pyrazofurin treatment, the largest changes were in the CTP, UTP and dCTP pools, but substantial decreases also occurred in the dGTP and dTTP pools; these decreased to 21 and 41% of control respectively. Again as with pyrazofurin treatment, the growth rate was unaffected after 6 hr of treatment, but the viability had decreased to 67% of control. Between 6 and 22 hr both the growth rate and viability declined to approximately 40% of control.

Both pyrazofurin and PALA inhibited pyrimidine synthesis *de novo* and, although the results from the two drugs were qualitatively similar, there were quantitative differences between them; thus, under pyrazofurin treatment, a growth rate of 17% of control corresponded to a cloning efficiency of 41% of control, whereas under PALA treatment a growth rate of 40% of control corresponded to 41% cloning efficiency. In addition, the minimum value for the dGTP pool size was 42% of control with pyrazofurin treatment, twice the minimum value achieved with PALA treatment. Pyrazofurin was much more potent than PALA in CHO cells; 0.2 μM pyrazofurin reduced the UTP pool to 25% and 10% at 2 and 22 hr of treatment, respectively, while 500 μM PALA reduced the UTP pool to 62 and 9% of control at 2 and 22 hr respectively.

Table 6. Nucleotide pools, growth rate, and viability during treatment of CHO cells with 500 μM PALA*

Treatment time (hr)	ATP	GTP	CTP	UTP	dATP	dGTP	dCTP	dTTP	Growth rate	Cloning efficiency
	(% of control)									
2	96	103	64	62	96	79	59	79	ND	109
4	112	110	43	34	103	52	40	81	ND	82
6	110	107	24	19	94	51	25	77	101	67
22	88	132	9	9	78	21	13	41	40	41

* Control nucleotide pools are reported in Table 2. ND = not determined.

Unlike pyrazofurin, PALA caused no change in cell cycle distribution at 6 hr of treatment, perhaps because of the slower rate of decrease in the concentrations of CTP, UTP and dCTP in PALA-treated cells (Fig. 1, g, h, and i). By 22 hr, there was a small decrease in the proportion of cells in the late S and G_2 phases with a corresponding increase in G_1 or early S.

There was little (40%) increase in modal cell volume even after 22 hr of treatment.

Thymidine. To determine the effects of varying mainly deoxyribonucleotide concentrations on cell growth rate and viability, cells were treated with 2 mM thymidine (Table 7). The main effects observed were very large increases in dTTP and dGTP, and large decreases in dCTP concentrations. Although 2 hr of treatment produced lower dCTP levels than were achieved at any time with pyrazofurin or PALA, the minimum growth rate achieved was still 63% of control, and the viability was still 86% of control after 22 hr of treatment. As well, it should be noted that 100 μM thymidine (which is not growth inhibitory), resulted in dTTP and dGTP concentrations of 500 and 200% of control, respectively, and dCTP concentrations of 12% of control; this was as low as the lowest level of dCTP achieved by treatment with pyrazofurin or PALA.

As shown in Fig. 1 (k and l), 2.0 mM thymidine inhibited the progression of the cells through the cell cycle with most of the cells being in G_1 and early S phase after 6 hr of treatment; however, between 6 and 22 hr the cells obviously overcame the block because by 22 hr the cell cycle distribution was similar to control with only a slightly smaller proportion of cells in the G_1 and G_2 phases.

After 6 hr of treatment, there was no change in the modal cell volume of the population, although substantial synchronization had occurred; however, by 22 hr the modal cell volume was approximately 180% of control.

DISCUSSION

The relationship between nucleotide concentrations and biological parameters appeared to vary both with respect to the nucleotide changes involved and with respect to the biological parameter studied. However, inhibition of cell growth could be correlated, at least in general terms, with decreases in the concentration of one or another ribo- or deoxyribonucleoside triphosphate. Thus, treatment with a growth inhibitory concentration of mycophenolic acid resulted in substantial decreases in both the GTP and dGTP pools and, after 2 hr of treatment with 2 μM mycophenolic acid, DNA and RNA syntheses were inhibited by 80 and 73% respectively. (To ensure that the measured rates of nucleic acid synthesis were independent of differences in nucleotide concentrations and radioactive precursor metabolism between control and drug-treated cells, the rates of radioactive precursor incorporation into nucleic acids were corrected for the specific activity of the immediate DNA or RNA precursor. The fact that similar results were obtained using several precursors suggests that the method is valid.)

The growth inhibition caused by pyrazofurin and PALA could also be rationalized in terms of an inhibition of DNA and RNA syntheses. Thus, after 2 hr of treatment with 0.2 μM pyrazofurin, DNA and RNA syntheses were inhibited by 6 and 50% respec-

Table 7. Nucleotide pools, growth rate, and viability during treatment of CHO cells with 2.0 mM thymidine

Treatment time (hr)	ATP	GTP	CTP	UTP	dATP	dGTP	dCTP	dTTP	Growth rate	Cloning efficiency*
	(% of control)									
2	48	82	84	84	92	269	5	2000	ND	ND
4	69	77	73	72	91	367	4	2164	ND	ND
6	91	97	88	95	122	514	3	2745	63	101
22	145	141	111	102	146	741	3	3692	63	86

* Cloning efficiency and nucleotide pool sizes were determined in separate experiments. The growth rates in the experiments in which nucleotide pools and cloning efficiency were measured were 48 and 63% respectively. Control nucleotide concentrations are reported in Table 2. ND = not determined.

tively. Finally, the slight inhibition of growth caused by 2.0 mM thymidine could be a result of an inhibition of DNA synthesis caused by the large decrease in the concentration of dCTP [3, 12–14], although it is also possible that the elevated dTTP and dGTP inhibited DNA synthesis directly or indirectly [15, 16]. The fact that 100 μM thymidine caused large changes in the concentrations of dTTP, dGTP and dCTP without inhibiting growth may indicate that these cells were relatively insensitive to changes in deoxyribonucleotide concentrations in the absence of changes in ribonucleotide concentrations.

The effects of the various treatment conditions on progression through the cell cycle are not as readily explained in terms of changes in nucleotide concentrations. Thus, mycophenolic acid caused an accumulation of cells in late G_1 or early S phase, while pyrazofurin caused little synchronization under strongly growth inhibitory conditions. PALA also caused little synchronization under growth inhibitory conditions. These results may suggest that a decrease in the concentrations of GTP and dGTP inhibited a specific step in the progression of cells through the cell cycle, while a decrease in the concentration of pyrimidine ribo- and deoxyribonucleoside triphosphates caused a relatively non-specific inhibition of progression through the cell cycle.

Thymidine, however, caused substantial synchronization at early treatment times, but much less synchronization at later times. This change in the degree of synchronization corresponded to relatively minor changes in nucleotide concentrations, and further study will be necessary to determine which time-dependent process allowed the cells to overcome the specific block in progression through the cell cycle.

The relationship between nucleotide concentrations and loss of cell viability was complex. First, in no case studied were altered nucleotide concentrations associated with decreased dye exclusion or with altered "energy metabolism", as assessed by the ATP:ADP ratio. A reasonable interpretation of these results is that at the time of cloning all the cells were metabolically healthy, but that the cells with low cloning efficiency had suffered permanent damage to their reproductive capacities during drug treatment.

Although in the case of mycophenolic acid treatment the loss of viability correlated with increases in dTTP concentrations, such a simple cause and effect relationship is unlikely; thus, much larger increases in dTTP, produced during thymidine treatment, had little effect on cloning efficiency. No other correlations of nucleotide concentration changes with cloning efficiency were observed, and it seems likely that cell death was related not only to changes in the concentration or one or another nucleotide, but also to the duration of abnormal imbalances in the nucleotide pool.

Lowe *et al.* [1] concluded that mycophenolic acid induced "unbalanced growth" in L5178Y cells because protein and RNA syntheses were inhibited less than DNA synthesis. This explanation does not apply to cell death in CHO cells induced by mycophenolic acid, since RNA and DNA syntheses were inhibited by similar amounts. As well, the phenomenon of "unbalanced growth" resulting in cell death is in itself not understood. Clearly, further studies of the relationship between nucleotide pool size changes and cell death are required.

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