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Purine Nucleotide Metabolism and Nucleotide Pool Sizes in Synchronized Lymphoma L5178Y Cells[1]

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SUMMARY

Mouse lymphoma L5178Y cells in culture were synchronized by exposure to thymidine and Colcemid. At four times during the cell cycle, various aspects of purine metabolism were examined with the use of radioactive purine bases. In all experiments the uptake and conversion of bases to nucleotides was rate limiting for ribonucleoside triphosphate synthesis. Cell cycle variations in the apparent enzyme activities of the purine phosphoribosyltransferases, adenosine monophosphate deaminase and guanosine monophosphate reductase, were observed. Purine and pyrimidine nucleotide pool sizes were determined by high-pressure liquid chromatography. After correction for increases in cell volume, it was observed that nicotinamide adenine dinucleotide, adenosine triphosphate, and guanosine triphosphate concentrations remained relatively constant throughout the cell cycle, whereas uridine diphosphate-glucose, cytidine triphosphate, and uridine triphosphate concentrations appeared to decrease in the S and G_2 phases.

INTRODUCTION

A delayed lethal effect of 6-mercaptopurine on cultured mouse lymphoma L5178Y cells has recently been reported (16), and it was concluded that this implies involvement of 6-mercaptopurine with macromolecular synthesis. Consistent with this suggestion was the observation that the 6-mercaptopurine toxicity was limited primarily to the S phase of synchronized lymphoma L5178Y cells (A. R. P. Paterson, unpublished observations). However, it was of interest to examine the possibility that this cell cycle specificity of 6-mercaptopurine might also be related to variation in the activities of enzymes of purine metabolism or of concentrations of purine nucleotides through the cell cycle. As there was very little information available as to such variations in mammalian cells, we therefore examined various aspects of purine metabolism, using synchronized lymphoma L5178Y cells, by utilizing radioactively labeled purine bases, while purine and pyrimidine nucleotide pools were determined directly by means of high-pressure liquid chromatography.

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MATERIALS AND METHODS

Cell Cultures. Mouse lymphoma L5178Y cells were routinely grown in suspension cultures with Fischer's medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% horse serum, streptomycin (100 μg/ml), and penicillin (100 units/ml) (17). Cell numbers were determined with Coulter Model F or B electronic particle counters. The cells were synchronized by 5 hr of exposure to 2 mM thymidine followed by 5 hr of exposure to 0.02 μg Colcemid per ml (Grand Island Biological Co.). This procedure is similar to that described by Doida and Okada (9). After resuspension in fresh medium, between 70 and 80% of the cells present underwent synchronous division (Chart 1).

Incubation with Purine Bases. Adenine-8-^{14}C (39.3 mCi/mmole), guanine-8-^{14}C (41.8 mCi/mmole), and hypoxanthine-8-^{14}C (49.4 mCi/mmole) were obtained from Schwarz/Mann, Orangeburg, N. Y. Duplicate 2.5-ml culture samples were transferred to stoppered 16- x 100-mm tubes and maintained at 37° for 20 min; radioactive purine bases were then added. We terminated incubations 30 min later by pouring the cultures into chilled centrifuge tubes, centrifuging down the cells in a clinical centrifuge, and extracting the cells with 0.010 ml of 0.4 M perchloric acid. After neutralization with 0.010 ml of 7 M KOH, the entire sample was spotted on polyethyleneimine-cellulose plates (Polygram Cel 300 PEI; Brinkman Instruments, Westbury, N. Y.). The purine ribo- and deoxyribonucleotides were separated by 2-dimensional chromatography (14) by a modification of the method of Crabtree and Henderson (8).

Analysis of Acid-soluble Nucleotides. Portions of synchronous lymphoma L5178Y cultures containing about 1.5×10^7 cells were taken at various times and poured into cooled centrifuge tubes. After 3 min in an ice-water bath with frequent mixing, the cells were collected by centrifugation and extracted with 0.3 ml of 0.4 M perchloric acid for 15 min at 4°. The supernatant was removed after centrifugation, and the pellet was reextracted with 0.1 ml of 0.4 M perchloric acid. The combined supernatants were neutralized to between pH 6 and 7 with 7 N KOH. The $KClO_4$ was removed by centrifugation, and the extract was concentrated to about 0.15 ml by 10% CO_2 gently bubbled through the solution at 4°. A known amount of UTP-^{14}C was added with the initial perchloric acid so that recoveries of acid-soluble nucleotides could be determined from the

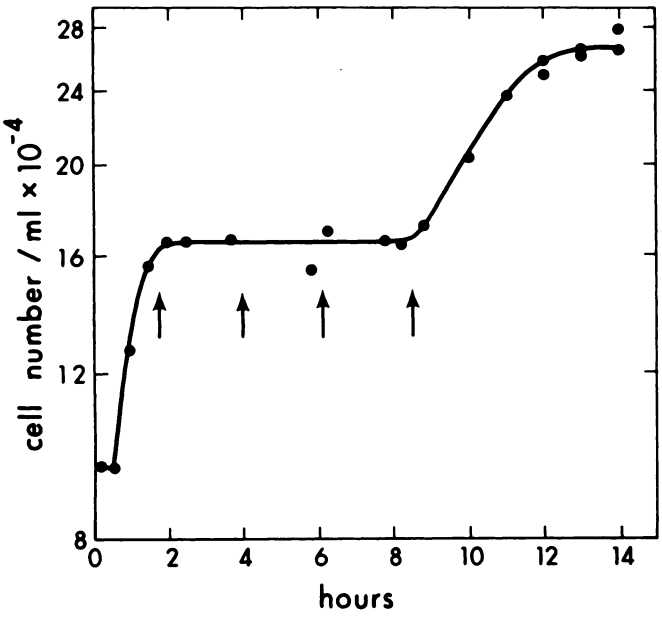

Chart 1. Increase in cell number of synchronized L5178Y cells with time. After the thymidine and Colcemid treatments, the cells were resuspended at zero time in fresh medium to a concentration of about 100,000 cells/ml. Cell numbers were determined with a Coulter counter. *Arrows*, times at which cells were used for the purine base studies or the acid-soluble nucleotide determinations.

final volume (determined by weight) and the recovered radioactivity. Using a triphosphate as a marker also provided an estimate of the extent of any breakdown of triphosphates due to the extraction or storage conditions. Essentially no breakdown was observed during extraction or storage of the frozen neutralized extracts for 24 hr.

Chromatography of the acid-soluble extracts was performed with a Varian Aerograph LCS-1000 liquid chromatograph with a 3-m capillary column packed with Varian PA-38 pellicular anion-exchange resin. The column effluent was monitored at 254 nm with the use of a low-volume (8 μl) flow cell with a 1-cm light path and a dual-pen recorder set for full-scale deflections of 0.002 and 0.008 absorbance unit. The acid-soluble nucleotides were separated, utilizing a linear gradient of potassium phosphate and potassium chloride at pH 4.5, essentially as described by Brown (5). The radioactivity profile of the UTP-^{14}C marker was determined by collecting 1-min samples and counting the entire sample in Bray's phosphor solution (1). The individual nucleotides were quantitated by comparing the peak areas, which were determined by planimetry, with a series of standard areas obtained with authentic nucleotides.

RESULTS

The labeling of ATP and GTP from radioactive bases in asynchronous lymphoma cells was linear for 30 min. Chart 2 shows the relationship between total nucleotide synthesis and purine base concentration for exponentially growing lymphoma L5178Y cells. Nucleotide synthesis from hypoxanthine and guanine was near maximal at 40 μM, while that for adenine was still not saturated at 50 μM. Because adenine is markedly toxic to L5178Y cells in culture at concentrations above 20 μM, this concentration of all 3 purines was chosen for the studies of synchronous cells.

The metabolism of the purine bases in the culture medium alone was examined by incubation of 20 μM adenine, guanine, or hypoxanthine for 30 min with medium without cells. The purine bases were isolated by column chromatography on Dowex 50 and by subsequent 2-dimensional chromatography on cellulose thin layers (8). In 30 min, 80% of the guanine originally present was converted to xanthine and 3% of the hypoxanthine was converted to xanthine, but there was no detectable metabolism of the adenine. The guanine deaminase activity has been attributed to the horse serum present in the culture medium.

Samples from a synchronous lymphoma L5178Y culture were taken at the 4 times shown on Chart 1 which corresponded to periods in the G_1, early S, late S, and G_2 phases, as indicated by thymidine-labeling experiments (S. C. Kim and A. R. P. Paterson, unpublished observations). The G_1 phase sample was taken just after the increase in cell number had reached a plateau, which was approximately 2 hr after the release of the Colcemid block. The subsequent samples were taken at 2-hr intervals.

Total nucleotide synthesis from each purine base during the 4 periods is shown in Table 1. Nucleotide synthesis from adenine increased to a maximum during the late S-phase period and then declined in the G_2-phase period. Extents of nucleotide synthesis from hypoxanthine and guanine were parallel but did not exhibit the magnitude of variation seen with adenine.

The synthesis of individual nucleotides, expressed in nmoles/10^9 cells, for the four 30-min labeling periods is also presented in Table 1. Most of the acid-soluble radio-

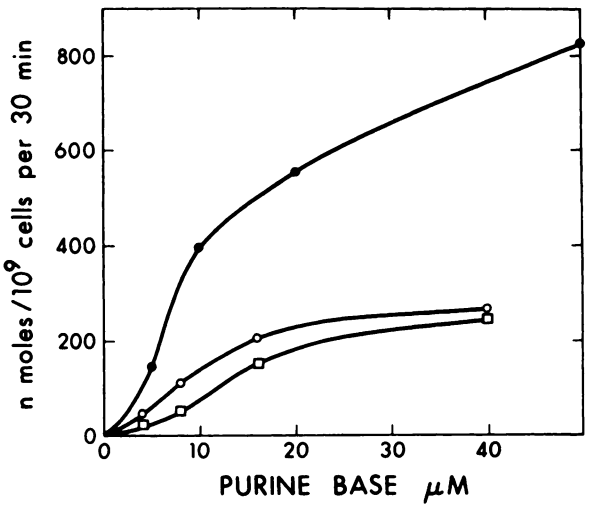

Chart 2. Total nucleotide synthesis from purine bases in asynchronous L5178Y cultures. Cultures (2.5 ml) of exponentially growing cells (141,800 cells/ml) were incubated for 30 min with the indicated concentrations of radioactively labeled adenine (●), guanine (□), or hypoxanthine (○). The total nucleotides synthesized was determined after polyethyleneimine-cellulose thin-layer chromatography.

Table 1

Nucleotides synthesized from purine bases

Synchronized L5178Y cells were incubated at various times in the cell cycle for 30 min with either 20 μM radioactively labeled adenine, guanine, or hypoxanthine. The neutralized acid-soluble extracts were chromatographed on polyethyleneimine-cellulose thin layers, and the extent of incorporation in the individual purine nucleotides was determined. Each value is the average of 2 determinations. The 4 time periods studied are shown in Chart 1.

Nucleotide	nmoles/30 min/10^6 cells											
	Adenine				Hypoxanthine				Guanine[a]			
	G_1	Early S	Late S	G_2	G_1	Early S	Late S	G_2	G_1	Early S	Late S	G_2
Total	363	659	734	557	165	216	196	224	107	137	126	128
ATP	286	545	635	465	94.7	126	110	131	1.7	14.6	2.6	2.2
ADP	46.4	66.7	56.9	53.0	12.1	22.2	22.3	23.1	0.5	3.0	1.3	0.7
AMP	15.1	15.5	8.9	7.7	1.8	4.1	9.9	6.2	—[b]	1.5	1.1	0.2
dATP	3.9	7.6	11.7	3.9	0.7	1.4	2.8	0.6				
dADP	—	0.3	0.3	0.1	0.2	0.1	0.1	0.3				
dAMP	1.5	1.4	0.3	0.8	0.3	1.2	—	0.3				
GTP	6.7	15.3	17.0	11.2	47.0	49.2	38.5	46.8	81.9	102	104	104
GDP	1.7	4.0	1.3	4.5	6.7	8.3	8.6	9.0	17.6	12.4	12.6	16.8
GMP	—	—	—	—	0.3	1.6	1.7	1.4	3.8	0.7	3.5	3.0
dGTP	0.5	1.1	1.3	0.6	0.5	0.9	0.6	0.6				
dGDP	—	—	—	—	—	—	—	—				
dGMP	—	—	—	—	0.1	0.2	0.1	0.1				
IMP	1.6	2.0	1.5	10.5	0.7	0.3	0.7	4.6	0.7	1.7	1.2	0.3
XMP	—	—	—	—	—	—	0.3	0.3	0.8	0.8	—	1.0

[a] Deoxyribonucleotides not determined with guanine labeling.

[b] —, no detectable levels of these nucleotides.

activity accumulated in the ribonucleoside triphosphates, namely ATP and GTP. The level of incorporation into the nucleoside monophosphates and diphosphates remained low, which indicates that uptake and conversion of bases to nucleotides was rate limiting for ribonucleoside triphosphate synthesis. The radioactivity found in dATP and dGTP was generally 1 to 2% of that found in ATP and GTP. The incorporation of the labeled bases into RNA and DNA was less than 5% of that into the acid-soluble fraction and was disregarded in the following analysis.

By assuming a simplified unidirectional pathway of metabolism for the synthesis of nucleoside triphosphates from each purine base, apparent activities can be calculated for several of the reactions of purine metabolism (14, 15). The apparent activity for a given step is represented by the sum of the radioactivity in all ribonucleotide and deoxyribonucleotide components that occur further along the pathway from the step in question. These results are shown in Table 2, together with the ratios of processes having AMP, GMP, or IMP as common substrates. The apparent rates of the phosphoribosyltransferases equal total nucleotide synthesis. The apparent activity of AMP deaminase increased from the G_1-phase period through the G_2-phase period, and the lowest AMP kinase/AMP deaminase ratio was in the G_2-phase period. The apparent activity of guanylate reductase was greatest in early S phase, which also had the lowest GMP kinase/GMP reductase ratio. Variation in the apparent activities of adenylsuccinate synthetase plus lyase and IMP dehydrogenase was small and reflected the changes in hypoxanthine phosphoribosyltransferase.

Apparent activities of ribonucleotide reductase have not been calculated, since the turnover of deoxyribonucleotide pools for DNA synthesis is rapid (13) and the pool sizes are small (2, 12). As shown in Table 1, the deoxyribonucleotide levels were highest during the S-phase periods when either adenine or hypoxanthine was used as precursor.

Chart 3 shows a typical elution profile of an acid-soluble extract. The concentrations of individual nucleotides analyzed by such methods are shown in Table 3 and are the averages of 5 experiments. Only upper limits could be assigned to UDP and GDP due to their low levels. The purine and pyrimidine monophosphates were present in only trace amounts.

The mean cell volumes, as determined with a Model B Coulter counter which was standardized against pollen grains of known size, were 920, 1050, 1175, and 1350 cu μm for the G_1, early S, late S, and G_2 samples, respectively. Plots of these values show that cell volume is increasing exponentially rather than linearly or in some discontinuous manner. Table 4 shows the relative concentrations of each nucleotide throughout the cell cycle after correction has been made for these changes in cell volume.

DISCUSSION

The results of this study do not support the hypothesis that the S-phase toxicity of 6-mercaptopurine toward lymphoma L5178Y cells is due to variation in either the activity of some enzyme of purine metabolism or in ribonucleoside phosphate pool sizes through the cell cycle. It

Table 2

Apparent enzyme activities during cell cycle

The apparent enzyme activities are determined as described in the text. Activities expressed are the averages of 2 determinations.

Precursor	nmoles/30 min/10^6 cells			
	G_1	Early S	Late S	G_2
Adenine				
Adenine phosphoribosyltransferase	363	659	734	557
AMP kinase	338	621	704	523
AMP deaminase	11	22	21	27
Hypoxanthine				
Hypoxanthine, phosphoribosyltransferase	165	216	196	224
Adenylosuccinate synthetase plus lyase	110	155	145	162
IMP dehydrogenase	55	60	50	58
Guanine				
Guanine phosphoribosyltransferase	107	137	126	128
GMP kinase	100	114	117	121
GMP reductase	4	22	6	4
Ratio of apparent activities				
AMP kinase/AMP deaminase	31	28	34	19
Adenylsuccinate synthetase plus lyase/IMP dehydrogenase	2.0	2.6	2.9	2.8
GMP kinase/GMP reductase	25	5	20	30

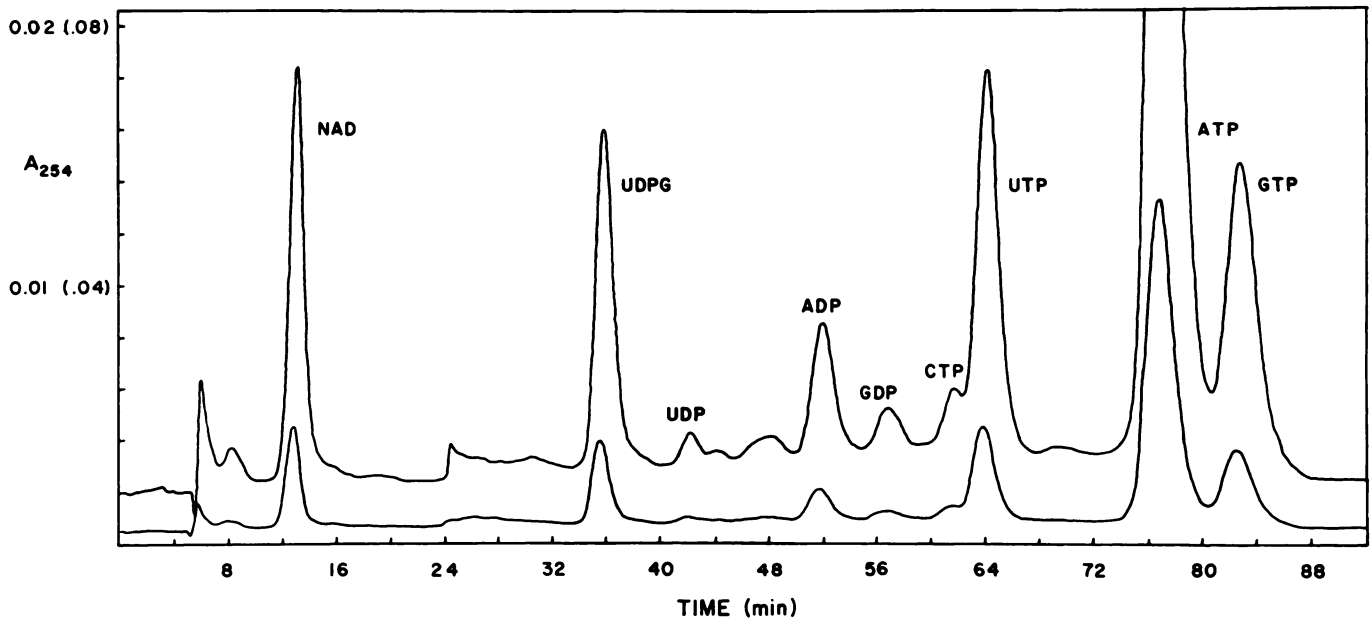

Chart 3. Anion chromatography of a 10-μl sample of an acid-soluble extract of G_1-phase L5178Y cells. The *upper curve* is at a sensitivity of 0.02 absorbance unit full scale, while the *lower curve* is at 0.08 A units full scale. *UDPG*, UDP-glucose.

would thus appear more likely that the delayed lethal effect of 6-mercaptopurine on lymphoma L5178Y cells (16) is due to an effect on DNA rather than on RNA synthesis or is related to the incorporation of some metabolite of 6-mercaptopurine into DNA.

The observation that there was no accumulation of purine nucleoside monophosphates or diphosphates indicates that the uptake and conversion of purine bases to nucleoside monophosphates is the rate-limiting step for purine nucleoside triphosphate synthesis from purine bases. This rate-limiting monophosphate formation could conceivably be due to the amount of phosphoribosyltransferase activity present or to the availability of the 2nd substrate, namely, phosphoribosylpyrophosphate. However, assays with cell-free extracts of lymphoma L5178Y have shown that the rate of base uptake observed in these studies represents less than 5% of the total phosphoribosyltransferase activity (L. Brox, unpublished results). Presumably, then, it is either the availability of phosphoribosylpyrophosphate or purine base uptake that is the rate-limiting factor.

As L5178Y cells in culture grow in a medium lacking preformed purines, it is obvious that they can provide their entire purine requirement by *de novo* synthesis. A minimal rate for purine *de novo* synthesis may then be estimated

Table 3

Acid-soluble pool sizes of synchronized L5178Y cells

At the times indicated on Chart 1, samples of a synchronized culture were removed, extracted, and neutralized, and a 10-μl sample was chromatographed on a Varian LCS-1000 liquid chromatograph. Values are averages of 5 separate experiments.

Nucleotide	nmoles/10^6 cells			
	G_1	Early S	Late S	G_2
NAD	331	439	424	543
AMP	Trace	Trace	Trace	Trace
GMP	Trace	Trace	Trace	Trace
UDP-glucose	1290	1220	1450	1160
UDP	<50	<50	<50	<50
ADP	251	310	301	355
GDP	<50	<50	<50	<50
CTP	465	475	350	295
UTP	1680	1630	1465	1480
ATP	2430	3110	3175	3620
GTP	455	520	595	690

Table 4

Relative concentrations of various acid-soluble components

The amount of each nucleotide per cell is individually normalized for the increase in cell volume that occurs throughout the cell cycle.

Nucleotide	G_1	Early S	Late S	G_2
NAD	1.00	1.16	1.00	1.12
UDP-glucose	1.00	0.83	0.88	0.61
ADP	1.00	1.08	0.94	0.96
CTP	1.00	0.90	0.59	0.43
UTP	1.00	0.85	0.68	0.60
ATP	1.00	1.12	1.02	1.02
GTP	1.00	1.00	1.02	1.03

from the total purine content of the cell (RNA, DNA, and acid-soluble purine) and the doubling time. This calculation indicates that the rates of nucleotide formation from purine bases observed in these studies of synchronous cells are about 10 to 15% of the average rate of purine synthesis required for cell growth.

As might have been expected, the highest amounts of radioactive dATP and dGTP were observed during the 2 S-phase labeling periods. The radioactivity found in these deoxytriphosphates was generally 1 to 2% of that found in ATP and GTP, which is consistent with other reports on triphosphate levels in mammalian cells (2, 7, 12, 18).

Table 4 shows that the concentrations of NAD, ADP, ATP, and GTP remain essentially constant throughout the cell cycle. It has previously been reported that the ATP concentration (as described by luciferase assays) in synchronous populations of Chinese hamster cells also remains essentially constant throughout the cell cycle (6). In synchronous *Escherichia coli* cultures that were uniformly labeled with inorganic ^{32}P, Huzyk and Clark (11) reported increases of perhaps 25% in the ATP and GTP concentrations just prior to cell division. It would thus appear that the concentrations of intracellular purine nucleotides remain more or less constant throughout the cell cycle, as indicated by results with the use of 3 different cell types. It would, however, be interesting to have such information on some mammalian line in which the DNA synthetic phase did not represent such a large proportion of the entire cell cycle, as is the case with lymphoma L5178Y cells. The feasibility of such studies on cultured human lymphoblasts is currently under investigation.

In contrast, the pyrimidine triphosphates and UDP-glucose appear to be at their highest concentrations in the G_1 phase, and there are well-documented instances of cell cycle variations in the apparent activities of various enzymes of pyrimidine metabolism (3, 4, 10). The high levels of UTP and hence of UDP-sugars early in the cell cycle may be related to the synthesis of cell membrane material.

The extremely low levels of both purine and pyrimidine monophosphates are also of interest. It is apparent that any purine or pyrimidine antimetabolite which accumulates to any extent as the monophosphate may easily have a concentration equal to or greater than the naturally occurring monophosphates and hence may have profound effects on the overall metabolism of the cell.

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