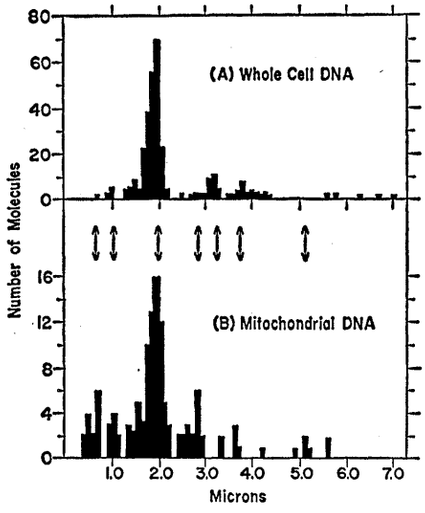

Fig. 3. Histograms of lengths of yeast circular DNA. Circles in DNA extracted from whole yeast cells (A) or from mitochondria prior to subfractionation in CsCl (B). Double-ended arrows indicate lengths of high-frequency classes.

material had a density of 1.701—slightly heavier than nuclear DNA; it may represent fragments of DNA that, while short enough to pass a nitrocellulose filter, still have sufficient denatured regions to make them denser. Another possibility is that this is 100-percent renatured DNA derived from the gamma or heavier satellite DNA present in yeast (*12, 13*). Electron microscopy showed that this filtrate contained both circles and short renatured rods; the circles were expected from the tendency of circular DNA to be renatured readily, but the composition of the short rods is unknown.

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Pyrimidine Nucleotide Synthesis: Regulatory Control during Transformation of Lymphocytes in vitro

Abstract. *The incorporation of uridine into RNA in lymphocytes undergoing transformation induced by phytohemagglutinin parallels its incorporation into the intracellular pool of acid-soluble nucleotides and coincides with a 20-fold increase in the specific activity of uridine kinase. This increase is dependent upon synthesis of both RNA and protein and is subject to both repression and end-product inhibition by cytidine.*

Lymphocytes from human blood undergo a striking morphologic transformation upon culture in the presence of phytohemagglutinin (PHA), a protein extracted from the kidney bean, *Phaseolus vulgaris* (*1*). The earliest biochemical changes preceding this transformation include an exponential increase in incorporation of uridine into RNA (*2*) and an increase in both the acetylation of histones (*3*) and the phosphorylation of histones and lipoproteins (*4*). Somewhat later, cells in culture produce gamma globulin (*5*), a variety of cellular enzymes active in glycolysis and oxidative phosphorylation (*6*), and a substance similar to interferon (*7*). The suggestion has been made that the process of transformation induced by PHA can be regarded as a nonspecific process of extensive gene activation (*4*).

I find that uridine kinase activity is induced in cells stimulated by PHA and that this process is subject to the types of regulatory control mechanisms of repression and end-product inhibition previously described for bacterial and mammalian cells (*8*).

Lymphocytes from human blood were purified by (i) a preliminary sedimentation by gravity after the addition of plasmagel (an amount equal to one-fourth the volume of blood) (*9*) to remove the bulk amount of erythrocytes; (ii) adsorption of the polymorphonuclear and other phagocytic cells onto a column of nylon fibers (*10*); and (iii) repeated sedimentation, by gravity, of samples concentrated by centrifugation to facilitate rouleaux formation of the erythrocytes. The final preparations, as judged from Wright staining, had only two polymorphonuclear and 8 to 20 red blood cells per 100 lymphocytes. The cells were cultured in Eagle's minimal essential medium (MEM) for suspension culture (supplemented with 15 percent fetal calf serum, glutamine, penicillin, and streptomycin) in a carbon dioxide

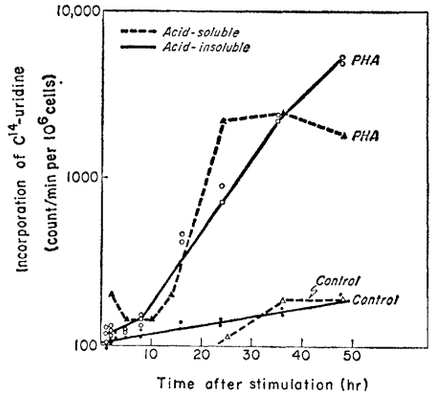

Fig. 1. Kinetics of ^{14}C-uridine incorporation into the intracellular pool of acid-soluble nucleotides and into RNA. Lymphocytes (5×10^6) in 2 ml of supplemented Eagle's MEM were incubated with 3.6 mμmole of ^{14}C-uridine (27.6 mc/mmole) for 2 hours at various times after the addition of PHA. Control cultures were labeled at the same time. The radioactivity in the acid-precipitated fraction retained on Millipore filters was determined in a liquid scintillation counter. The counts were normalized to 10^6 cells. The filtrate was collected quantitatively, and the radioactivity was determined from samples. Incorporation into RNA is indicated by solid lines; incorporation into the acid-soluble pool, by the dashed line.

incubator adjusted to maintain a *p*H of 7.4 with the carbonate-bicarbonate buffer. Treated cultures contained 5 μg of PHA-P (Difco Preparation P) per 10^6 lymphocytes.

The number of cells in culture was determined electronically in well-dispersed samples with a Coulter counter, and by assay of the DNA content of samples containing about 10^6 cells with a Burton diphenylamine assay (*11*) for deoxypentose (10^6 lymphocytes contain 15.5 mμmole of DNA-deoxypentose). Under these conditions of culture, there is a 25 percent decrease (standard deviation = 11 percent) in the number of cells during the first 24 hours of incubation with PHA and no decrease in the number of cells in cultures without PHA. The percentage of cells that stain with the vital dye trypan blue ranged from 0 to 4 percent in both stimulated and unstimulated cultures.

Incorporation of ^{3}H-uridine or ^{3}H-orotic acid was determined in cells incubated with the appropriate radioactive presursor for an hour at various times after the addition of PHA. The cells were harvested by centrifugation at 800 rev/min for 8 minutes, washed by resuspension of the pellet in ice-cold glucose-(0.1 percent)—saline (0.9 percent) solution and homogenized in cold 10 percent trichloroacetic acid (TCA) by treatment with ultrasonic vibration. The material precipitated by the TCA was collected by filtration through a millipore disc (0.45-μ pore size), and a portion of the filtrate was used for determination of the rate of entry of labeled uridine into the total pool of acid-soluble nucleotides. The distribution of label in the nucleotides was determined by co-chromatography of concentrated TCA-extracts with added nucleotide as an optical density marker on two-dimensional thin layer polyethyleneimide-cellulose plates (*12*). Incorporation of ^{14}C-aspartic acid into RNA was measured by a modification of the above procedure. After the cells were disrupted in acid by ultrasonic vibration and the homogenate was centrifuged, the pellet was digested in 0.3*M* KOH for 1 hour at 37°C and reprecipitated with perchloric acid; a sample of the hydrolyzed RNA was counted. A Nuclear-Chicago Mark 1 liquid scintillation spectrometer equipped with an external standard was used to determine the radioactivity and the counting efficiency of the samples.

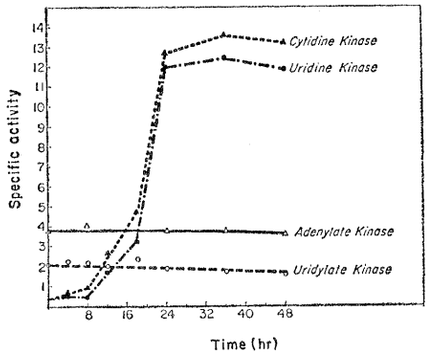

Fig. 2. Induction of nucleoside kinase activities during incubation with PHA. Homogenates of cultures containing 5 × 10^7 cells were prepared at each time point as described in the legend to Table 2. The activities of adenylate kinase and uridylate kinase were assayed essentially as described for the nucleoside kinases with two modifications: The reaction mixtures contained 6 mμmole of ^{3}H-adenosine-5′-monophosphate (4.2 c/mmole) or 12 mμmole of ^{3}H-uridine-5′-monophosphate (27.6 mc/mmole), respectively. Aliquots of the heated reaction mixture were streaked on polyethyleneimide-cellulose plastic thin-layer chromatography sheets and were developed in a LiCl gradient to achieve maximum separation between the phosphorylated nucleotides (*12*). The mixture was co-chromatographed with 15 mμmole of the appropriate nucleoside di- or triphosphate, and the reaction products were visually identified by ultraviolet light. The uridylate kinase activity was taken as the sum of the radioactivity appearing in the nucleoside di- and triphosphate spots. The specific activity is given as the millimicromoles of substrate used per 30 minutes per milligram of protein.

Cell extracts of samples containing 5 × 10^7 lymphocytes in 1 ml of a solution of 20 percent glycerol, 0.1*M* tris(hydroxymethyl)aminomethane (tris)-HCl, *p*H 7.4, and 0.001*M* 2-mercaptoethanol were made by treatment for 30 seconds with a Branson Sonicator. Assays for uridine, cytidine, uridylate, and adenylate kinases are described in the legend to Fig. 2. Protein concentrations were determined by the Lowry phenol reagent method (*11*). Specific activity of the kinases is expressed as the number of millimicromoles of substrate used in 30 minutes at 37°C per milligram of protein.

The rate of incorporation of labeled uridine into RNA increases nearly exponentially after stimulation with PHA (Fig. 1). At 24 hours of culture, the rate is some 14 to 26 times that of freshly isolated cells. The actual increase in rate differs over rather wide limits depending on the donor's cells, or on some as-yet unidentified serum factor. In contrast, the incorporation of nucleotide presursors by way of *de novo* pathway of pyrimidine nucleotide synthesis does not show such a striking increase (Table 1). The rate of incorporation of either ^{14}C-aspartic acid or ^{3}H-orotic acid into RNA is increased at best only about twofold. The rate of entry of labeled uridine into the pool of acid-soluble nucleotides (Fig. 1) parallels the rate of its incorporation into RNA. This finding suggests that the rate-limiting step in the incorporation of uridine into RNA may be the initial enzymatic step in the salvage pathway, the phosphorylation of uridine by uridine kinase. In support of this is the finding that in acid-soluble extracts of lymphocytes labeled with ^{14}C-uridine at 6 and 24 hours after incubation with PHA, 96 percent of the radioactivity is recovered in uridine triphosphate, uridine diphosphate, uridine monophosphate, and cytidine monophosphate on two-dimensional thin-layer chromatography on polyethyleneimide-cellulose; of this, 85 percent was uridine triphosphate (*13*). A low rate of incorporation of labeled uridine both into the acid soluble pool and into RNA in cells incubated without PHA occurs consistently (Fig. 1). This incorporation can be reduced but not entirely eliminated by incubation of the cells in autologous plasma and without antibiotics.

Table 1. Incorporation of pyrimidine nucleotide precursors into RNA of lymphocytes. Duplicate cultures of 10^7 cells were incubated in 2 ml of supplemented Eagle's MEM. To appropriate cultures, 600 mμmole of L-aspartic-^{14}C acid (167 mc/mmole), 350 mμmole of ^{3}H-orotic acid (140 mc/mmole), or 1.2 mμmole of ^{3}H-uridine (22 c/mmole) were added 1 hour before harvest. Homogenates of the acid-precipitated cultures were collected on Millipore filters as described in the text, and the radioactivity on the washed filter was determined.

Condition	Incorporation into RNA per hour per 10^7 cells (count/min)		
	^{14}C-aspartic acid	^{3}H-orotic acid	^{3}H-uridine
Control, 0 time	194	658	729
Control, 24 hours	225	984	1313
PHA, 24 hours	453	2169	26,000

The specific activity of uridine kinase in the homogenates increases 20-fold after stimulation (Fig. 2), the increase coinciding temporally with the major increase in uridine incorporation into both the nucleotide pool and RNA. Similarly, the specific activity of cytidine kinase also increases, but the ac-

tivities of uridylate and adenylate kinases remain constant. The greater activity of uridylate kinase present in unstimulated cells again indicates that uridine kinase may be the rate-limiting determinant. The increase in specific activity of uridine kinase is dependent on synthesis of both RNA and protein, since actinomycin D (1 μg/ml) and puromycin ($10^{-6}M$) inhibit the observed increase (Table 2).

That the induction of uridine kinase is not a direct and immediate consequence of PHA action is indicated by the demonstration that uridine utilization and uridine kinase formation are under regulatory processes of repression and end-product inhibition which operate independently of PHA. Cytidine at physiologic concentrations has a profound inhibitory effect on the incorporation of ^{3}H-uridine into lymphocytes 24 hours after their culture with PHA (Fig. 3). The inhibition of uridine incorporation is unique to cytidine. Cultures grown in $10^{-4}M$ concentrations of the nucleosides adenosine, deoxyadenosine, guanosine, inosine, deoxycytidine, or thymidine show no decrease in intracellular uptake of uridine. However, despite the inhibition of uridine incorporation and uridine kinase induction, morphologic criteria of transformation as judged from Wright stains of smears and incorporation of ^{3}H-adenosine or ^{3}H-cytidine into RNA indicate that transformation and RNA synthesis continue. Electron microscopic observations of the cells did show one striking feature; large numbers of granules thought to contain glycogen appeared early in the course of transformation (*12*). These granules were not seen in control cultures or seen in such large amounts in cells stimulated with PHA and grown in the absence of cytidine.

The inhibition in uridine incorporation is the result of two factors. The synthesis of uridine kinase is inhibited in stimulated cultures grown in $10^{-4}M$ cytidine. The cells in such cultures have only 50 percent of the uridine kinase activity of cells grown in the absence of cytidine (Table 2). In addition, uridine kinase activity in assays in vitro is completely inhibited by cytidine triphosphate at a concentration of $10^{-4}M$. Uridine has a similar and reciprocal effect on incorporation of ^{3}H-cytidine into cells, on inhibition of the induction of cytidine kinase during stimulation with PHA, and on the inhibition by uridine triphosphate of cytidine kinase activity in assays in vitro. Once the maximum level of uridine kinase activity is induced (14 hours), subsequent addition of cytidine does not alter the amounts of enzyme activity in the extracts.

These results are compatible with the interpretation that cytidine, or more likely a phosphorylated derivative of cytidine, functions as a co-repressor for synthesis of uridine kinase and that the primary action of PHA, although still obscure, results in a decrease in the concentration of this cytidine metabolite. Mechanisms which could cause the depletion of the intracellular concentrations of such a metabolite and be a more direct result of the action of PHA immediately suggest themselves: increased cellular permeability to nucleotides; increased utilization of cytidine triphosphate in RNA synthesis; increased utilization of cytidine diphosphate-choline in lipid synthesis. Whatever the primary mechanism, the decrease in intracellular concentration of the cytidine metabolite derepresses the cellular mechanism for the synthesis of uridine and cytidine kinases. These experiments provide strong evidence that in PHA stimulated cells the induction of uridine kinase is not the result of a direct gene activation by PHA.

Table 2. Specific activity of nucleoside kinases in sonically disrupted lymphocytes. Cultures containing 5×10^7 lymphocytes in 20 ml of supplemented Eagle's MEM in 50-ml conical tubes were grown for 24 hours with PHA and the specific nucleoside or inhibitor. After centrifugation, the cells were disrupted in 1 ml of an ice-cold solution of glycerol, tris, and mercaptoethanol. For assay of uridine kinase activity in the crude extract, 0.09 mg of protein was incubated in a reaction mixture containing 3.8 mμmole of uridine-^{14}C (27.6 mc/mmoles), 0.5 μmole of ATP, 10 μmole of $MgCl_2$, and 10 μmole of tris-HCl, *p*H 7.4, in a total volume of 0.18 ml, for 30 minutes at 37°C. The reaction was stopped by boiling for 3 minutes. Samples of each reaction mixture were streaked on polyethyleneimide-cellulose on plastic thin-layer chromatography sheets and developed in water. The radioactivity remaining at the origin represented the phosphorylated uridine nucleotides. I counted these by cutting out the origin and placing it directly in toluene, 2,5-diphenyloxazole and dimethyl 1-4-bis[2-(5-phenyloxazolyl)]-benzene scintillation solution. There was enzyme proportionality to 0.18 mg protein. Thus, with 0.045 mg of protein, 3450 count/min were incorporated into nucleotides; with 0.09 mg, 6990 count/min; and with 0.18 mg, 10,890 count/min; the reaction blank had 123 count/min. Cytidine kinase activity was measured similarly, with 12.5 mμmole of cytidine-^{14}C (20 mc/mole).

Culture condition	Substrate (mμmole) used per 30 minutes per milligram of protein	
	Uridine kinase	Cytidine kinase
0 time, control	0.5	0.4
24 hour, control	1.6	1.4
PHA	12.0	12.6
PHA, $10^{-4}M$ cytidine	4.5	10.4
PHA, $10^{-4}M$ uridine	6.1	7.1
PHA, $10^{-4}M$ deoxycytidine	11.4	12.9
PHA, actinomycin, 1 μg/ml	2.1	1.6
PHA, $10^{-6}M$ puromycin	1.8	1.9

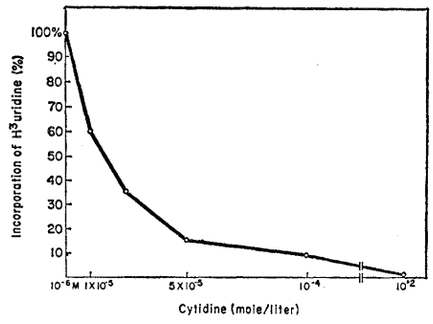

Fig. 3. Effects of cytidine on the incorporation of ^{3}H-uridine in lymphocytes stimulated by PHA. Duplicate cultures of 5×10^6 lymphocytes were incubated for 24 hours with 25 μg of PHA and the experimental amount of cytidine. At that time, 1.2 mμ mole of ^{3}H-uridine (22.0 c/mmole) was added, and the cells were incubated an additional hour. The radioactivity in the acid-precipitable fraction retained on a Millipore filter was determined and expressed as the percentage of the value of the culture containing no cytidine (26,000 count/min ± 6 percent).

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Transport and Phosphorylation as Factors in the Antitumor Action of Cytosine Arabinoside

Abstract. *Survival of mice bearing different transplantable leukemias and treated with cytosine arabinoside was compared with uptake and subsequent phosphorylation of the drug in vitro. Capacity for nucleotide formation was correlated with response and is apparently an important determinant of drug sensitivity. Drug uptake, although apparently mediated, was similar in all cell lines.*

We have reported that variation in uptake of the nonmetabolized drug methotrexate (*1*) and variations in capacity for phosphorylation of the freely diffusible drug 5-fluorouracil (*2*) were determining factors of drug response in leukemias in mice. We now find that cytosine arabinoside is not freely diffusible, but that the rate of intracellular phosphorylation—not uptake of the drug—is correlated with drug response (Fig. 1).

Cytosine arabinoside (1-β-D-arabinofuranosyl-cytosine) inhibits growth of certain animal (*3*) and human (*4*) neoplasms, presumably by competitively inhibiting reduction of cytidine ribotides to deoxyribotides (*5*). Another possible mode of the drug's action is its own incorporation into the DNA and RNA of mammalian cells (*6*). The ability of cytosine arabinoside to interrupt DNA synthesis was not considered to be related to incorporation of the drug into terminal positions of cell DNA (*7*). Phosphorylation of the drug to nucleotides was required for its antitumor action, and impaired drug phosphorylation was associated with development of drug resistance in cultured P5178Y leukemia cells of mice (*8*).

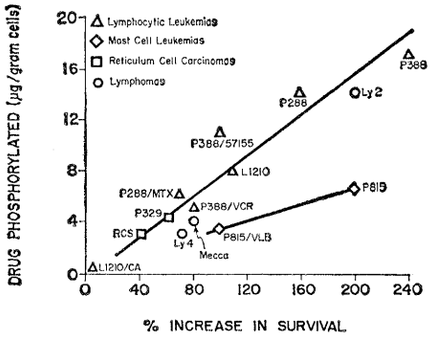

Fig. 1. Correlation between cytosine arabinoside phosphorylation in vitro and drug-promoted survival of tumor-bearing animals. Phosphorylation was determined by measurement of drug conversion to nucleotides. Results are shown in terms of micrograms of drug phosphorylated per gram of cells (wet weight) per 15-minute incubation at 37°C. Survival data were given by Wodinsky and Kensler (*9*). Each point represents cells from the tumor cell line indicated.

We examined 14 transplantable mouse leukemias which varied widely (*9*) in responsiveness to the drug (*1, 10*). Ascitic cells were removed from animals and suspended in buffer (*11*). Samples of 150 μl containing 7 to 8 mg of cells (wet weight) were incubated with 10 μg per milliliter of radioactive cytosine arabinoside or other nucleosides (*12*). Incubations were ended by chilling the tubes for 30 seconds. The cells were collected by centrifugation for 30 seconds at 150*g*, washed by suspension in ice-cold buffer, and collected by centrifugation. Uptake of drug and conversion to nucleotides were measured at this point by determining the total radioactivity in the cells. To determine the concentrations of labeled nucleotides in the cells, the pellets were again suspended in 250 μl of buffer at 37°C for 5 minutes and collected by centrifugation. All unmetabolized nucleoside was washed from the cells by this procedure; loss of labeled nucleotides was negligible. Cell radioactivity was measured by extracting intracellular components with 250 μl of 0.1*N* acetic acid at 60°C for 10 minutes. Cell debris, which contained negligible radioactivity, was removed by centrifugation, and a 200-μl fraction of the supernatant was used for radioactivity measurements (*13*).

Radioactive components of cell extracts were identified by paper chromatography. Drug nucleotides and unmetabolized drugs were extracted with perchloric acid, and the extracts were neutralized and concentrated (*2*). Diffusible and nondiffusible labeled cell components were identified with appropriate compounds as markers on chromatograms (*14*). Drug detoxification results from deamination of cytosine arabinoside to uracil arabinoside, an inactive product (*15*). Deaminase activity could not be found in L1210 or L1210/CA cells (*16*), nor did we obtain any chromatographic evidence of deamination of cytosine arabinoside by any cell line that we tested.

Of the four types of mouse leukemias tested, only the mast-cell leukemias showed significant sensitivity to cytosine arabinoside in the absence of a correspondingly high capacity for drug phosphorylation (Fig. 1). This may be related to our observation that rate of phosphorylation of other nucleosides by mast cells was relatively low (*17*).

Nucleotide formation in drug-resistant leukemias might be limited by impaired drug uptake. Therefore, it was important to show that cytosine arabinoside was not excluded from drug-resistant cell lines. We first studied uptake of the drug in L1210/CA, a line derived from L1210 by selection for drug resistance (*18*). Uptake of cytosine arabinoside by L1210/CA cells was essentially temperature-insensitive; a ratio of about 0.2 for intracellular to extracellular distribution of the drug was achieved within 3 minutes after the start of incubations (Fig. 2). This ratio was not increased by prolonging incubations. Accumulation of cytosine arabinoside by L1210 cells was highly temperature-sensitive and apparently concentrative (Fig. 2). But chromatographic examination of cell extracts showed that the labeled intracellular material accumulated at 37°C con-

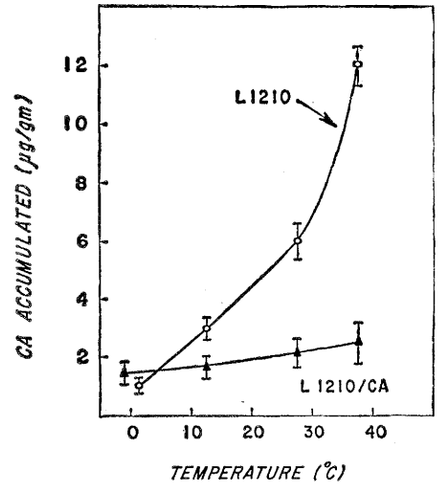

Fig. 2. Accumulation of cytosine arabinoside plus nucleotides of the drug by tumor cells as a function of temperature of incubation. Cells were incubated in medium containing 10 μg per milliliter of labeled drug for 15 minutes; the resulting intracellular accumulation is reported in terms of micrograms of drug per gram of cells (wet weight). After incubations at 37°C, $>$ 95 percent of the cell radioactivity in L1210/CA represented the unchanged drug; 70 percent of the radioactivity in L1210 represented drug nucleotides (*14*).