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METABOLISM OF 5-AMINO-1-β-D-RIBOFURANOSYL-IMIDAZOLE-4-CARBOXAMIDE AND RELATED FIVE-MEMBERED HETEROCYCLES TO 5′-TRIPHOSPHATES IN HUMAN BLOOD AND L5178Y CELLS

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Abstract—The facile metabolism of 5-amino-1-β-D-ribofuranosylimidazole-4-carboxamide (AICA ribonucleoside) and several of its structural analogs [5-fluoro-1-β-D-ribofuranosylimidazole-4-carboxamide (FICA ribonucleoside), pyrazofurin and ribavirin] to their corresponding 5′-triphosphates in human blood cells has been demonstrated *in vitro*. Evidence is presented that both the β- and α-anomeric forms of pyrazofurin nucleotides were present in the extracts of pyrazofurin-treated blood. Determination of the extent of incorporation of radioactivity from [U-^{14}C]D-glucose into analog ribonucleoside 5′-triphosphates formed in human blood cells indicated that AICA ribonucleoside and ribavirin were metabolized to their 5′-monophosphates mainly (>90 per cent) via a nucleoside kinase; however, FICA ribonucleoside appeared to be metabolized to its 5′-monophosphate both via a nucleoside kinase (*ca.* 67 per cent) and via phosphorolytic cleavage followed by a phosphoribosyltransferase-mediated reaction (*ca.* 33 per cent). The aglycones of AICA ribonucleoside and FICA ribonucleoside were also metabolized extensively to their corresponding ribonucleoside 5′-triphosphates. 5′-Aminoimidazole-4-thiocarboxamide and 3-aminopyrazole-4-thiocarboxamide were metabolized only slightly to their ribonucleoside 5′-triphosphates. [^{3}H]ribavirin was metabolized extensively to its 5′-triphosphate in L5178Y cells but was not detectably incorporated into RNA. Ribavirin caused a substantial decrease in the pool size of GTP in L5178Y cells and a concomitant increase in the pool size of both CTP and UTP.

The 5′-monophosphate of 5-amino-1-β-D-ribofuranosylimidazole-4-carboxamide (AICA* ribonucleoside) is a naturally occurring intermediate in the pathway of purine biosynthesis *de novo*. During the past few years, an impressive number of structural analogs of AICA ribonucleoside have been found to exhibit potentially useful therapeutic activities. Pyrazofurin, a C-nucleoside, possesses both antitumor and antiviral properties [1]. Ribavirin, in addition to its antiviral properties [2], has been reported to exhibit antitumor [3–5] and immunosuppressive activities [5, 6]. FICA ribonucleoside appears to closely resemble ribavirin in its spectrum of antiviral activity [7]. ThioAICA ribonucleoside [8] and bredinin [9–11] are each active as antitumor agents, while bredinin has also been found to have antiviral and immunosuppressive properties [9]. The chemical structures and names of these various compounds are shown in Fig. 1.

Progress in elucidating the biochemical mechanisms of action of these AICA-related compounds has been hindered by the limited knowledge currently available concerning their metabolism. Both AICA ribonucleoside and its aglycone are metabolized, albeit to a limited extent, to inosinate in human erythrocytes [12, 13] and also to ATP and GTP in rabbit erythrocytes [14]. AICA ribonucleoside has been reported to be a substrate for purine nucleoside phosphorylase isolated from beef liver [15]; however, AICA ribonucleoside was not cleaved phosphorolytically by intact rabbit erythrocytes [14], cells which contain high levels of purine nucleoside phosphorylase [16]. Adenosine kinase purified from cultured human tumor (H. Ep. 2) cells has been reported not to phosphorylate AICA ribonucleoside [17]. The aglycone of AICA ribonucleoside can be metabolized to its ribonucleoside 5′-monophosphate via adenine phosphoribosyltransferase [18–20]. Ribavirin has been shown to be metabolized to its 5′-monophosphate in rodent liver [2] by an enzyme tentatively identified as deoxyadenosine kinase [21]; no evidence of 5′-di- or -triphosphate metabolites of ribavirin was found [2]. Chemically synthesized pyrazofurin 5′-monophosphate has been shown to be an extremely potent inhibitor of orotidylate decarboxylase; the elevated levels of orotic acid and orotidine observed in body fluids of pyrazofurin-treated animals and humans suggest the phosphorylation of this drug *in vivo* and consequent inhibition of the pyrimidine biosynthetic pathway at orotidylate decarboxylase [1]. ThioAICA ribonucleoside is metabolized extensively to 6-mercaptopurine in mice, presumably by metabolism of thioAICA ribonucleoside to its 5′-monophosphate and subsequent conversion to 6-thioinosinate via the terminal steps of the purine biosynthetic pathway [22]. The observation that the growth inhibitory activity of bredinin can be reversed

* Abbreviations used: AICA, 5-aminoimidazole-4-carboxamide; FICA, 5-fluoroimidazole-4-carboxamide; PBS, phosphate-buffered saline; pyrazofurin, 3-β-D-ribofuranosyl-4-hydroxypyrazole-5-carboxamide; ribavirin, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide; and thioAICA, 5-aminoimidazole-4-thiocarboxamide.

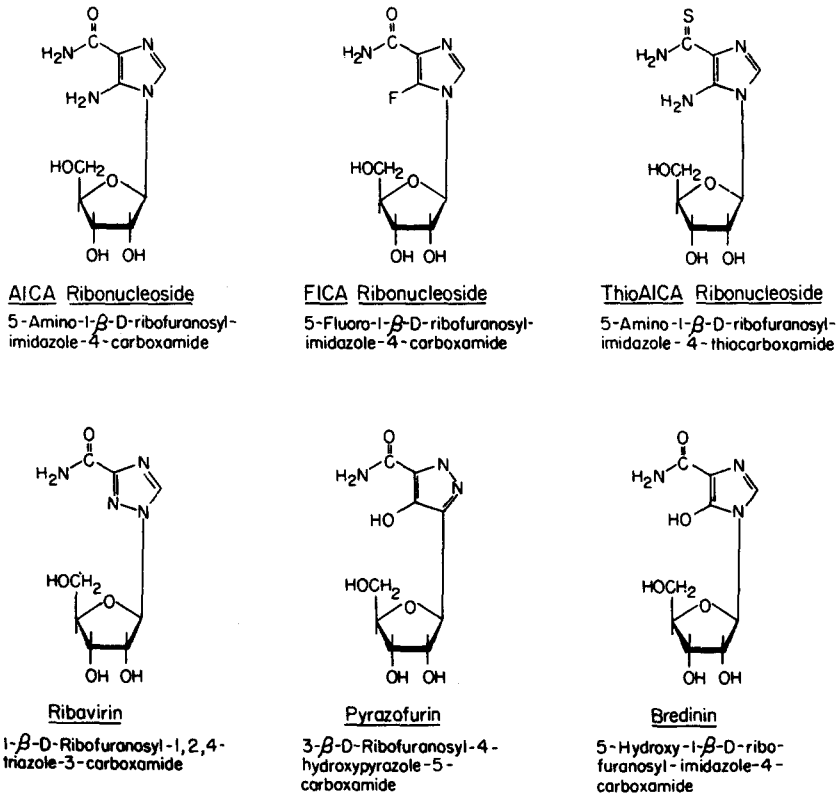

Fig. 1. Chemical structures and names of AICA ribonucleoside and several of its biologically active structural analogs.

by guanine-containing compounds suggests that this antibiotic, possibly as a 5'-phosphate metabolite, may function by inhibiting the biosynthesis of GMP [10, 11].

The present report documents the facile metabolism of AICA ribonucleoside and several of its structural analogs to their corresponding 5'-triphosphates in human blood cells *in vitro* and, in the case of ribavirin, in L5178Y cells. While this work was in progress, abstracts appeared describing the metabolic formation of 5'-polyphosphates of pyrazofurin [23] and ribavirin [24] in various mammalian cells.

MATERIALS AND METHODS

Materials. AICA, AICA ribonucleoside, ITP, ATP, GTP, penicillin G, streptomycin sulfate and snake venom (*Crotalus adamanteus*) phosphodiesterase were obtained from the Sigma Chemical Co. FICA and FICA ribonucleoside were generous gifts from Dr. Louis A. Cohen of the National Institutes of Health. Pyrazofurin was generously provided to our laboratories by Dr. Gerald E. Gutowski of Eli Lilly & Co. Ribavirin, both unlabeled and ^{3}H-labeled (sp. act. 100 mCi/m-mole), was purchased from ICN Life Sciences Group. 5-Aminoimidazole-4-thiocarboxamide (thioAICA), 5-amino-1,2,3-triazole-4-carboxamide, 5-aminoimidazole-4-thiocarboxylic acid methyl ester and 5-aminoimidazole-4-carboxhydrazide were synthesized by Dr. Janet L. Rideout and 3-aminopyrazole-4-thiocarboxamide and 3-aminopyrazole-4-carboxamide were synthesized by Dr. Gertrude B. Elion at the Wellcome Research Laboratories. Yeast hexokinase (140 units/mg) was a product of Boehringer Mannheim. [2-^{14}C]imidazole (sp. act. 3.5 mCi/m-mole) was obtained from Mallinckrodt Chemical Works. [5-^{14}C]3-amino-1,2,4-triazole (sp. act. 4.5 mCi/m-mole), Protosol and Econofluor were from New England Nuclear, and [8-^{3}H]cyclic AMP (sp. act. 27 Ci/m-mole) and *Escherichia coli B* transfer RNA were from Schwarz/Mann. [U-^{14}C]D-glucose (sp. act. 271 mCi/m-mole), [U-^{14}C]AMP (sp. act. 500 mCi/m-mole) and [5-^{3}H]uridine (sp. act. 26 Ci/m-mole) were products of Amersham/Searle. The [U-^{14}C]D-glucose was diluted isotopically with unlabeled D-glucose to a final specific activity of 0.42 mCi/m-mole prior to its use. Dulbecco's phosphate-buffered saline, Fischer's medium and dialyzed horse serum were from Grand Island Biological Co. and RPMI 1640 medium was from Flow Laboratories. Fischer's medium (F_{10P}) was prepared as described previously [25].

Cells. Human blood samples were withdrawn from healthy volunteers into heparinized syringes. L5178Y cells, harvested from suspension culture in Fischer's medium, were generously provided by Dr. Donald Clive of these laboratories.

Incubation and extraction of blood cells. Fresh heparinized whole blood was supplemented with 38 mM potassium phosphate (pH 7.4), 25 mM D-glucose, 0.5 mg/ml each of penicillin G and streptomycin sulfate, and saline or test compound, such that the final hematocrit was approximately 32. Incubations were carried out at 37° in a Dubnoff metabolic shak-

ing incubator with air as the gas phase. At the specified times, 2.0-ml samples of each incubation mixture were withdrawn and immediately mixed with 10 ml of cold 0.5 M perchloric acid which contained approximately 4×10^5 cpm of [U-^{14}C]AMP as a recovery marker. The resulting extracts were clarified by centrifugation and subsequent filtration through glasswool, neutralized with KOH, centrifuged to remove the insoluble potassium perchlorate, evaporated to dryness under reduced pressure (in a Buchler Evapo-Mix apparatus) and reconstituted in 400 μl of deionized water. These samples were stored at −20° until analyzed in the liquid chromatograph.

Analysis of cell extracts by high-performance anion-exchange chromatography. Acid-soluble extracts of human blood cells and L5178Y cells were analyzed in a Varian Aerograph model LCS-1000 high-pressure liquid chromatograph equipped with a Reeve Angel column (0.46 × 25 cm) containing the microparticulate anion-exchange resin Partisil-10 SAX. An auxiliary, variable wavelength u.v. detector (Schoeffel model SF770) was connected in series with the standard 254 nm detector to allow simultaneous monitoring of the column effluent at two different wavelengths with a Honeywell Electronik 194 two-pen recorder. For maximum sensitivity, the 5'-ribonucleotide metabolites of AICA ribonucleoside, FICA ribonucleoside, pyrazofurin, ribavirin, thioAICA, 3-aminopyrazole-4-thiocarboximade, 3-aminopyrazole-4-carboxamide and 5-aminoimidazole-4-carboxhydrazide were monitored at 267, 227, 262, 207, 325, 310, 252 and 270 nm, respectively, with the Schoeffel u.v. detector. Fifteen-μl samples of each cell extract were injected into the liquid chromatograph. The eluents were 0.02 M and 1.0 M potassium phosphate, both at pH 3.5. The starting volume of 0.02 M potassium phosphate in the mixing chamber was 30 ml. The column flow rate was 30 ml/hr and the flow rate of 1.0 M potassium phosphate into the mixing chamber was 20 ml/hr. A 15-min gradient delay was employed. The temperature of the column chamber was 30°.

Peaks of endogenous nucleotides were identified by comparison with the retention times of authentic standards and by their A_{254}/A_{280} absorbance ratios. Nucleotide concentrations in the cell extracts were calculated using response factors [ultraviolet peak area (in^2)/nmole of nucleotide] determined by injecting known amounts of authentic nucleotide standards into the liquid chromatograph under the standard operating conditions. AICA ribonucleoside, FICA ribonucleoside, pyrazofurin and ribavirin were used to determine the response factors for their respective nucleotide metabolites. A portion of each extract was monitored by liquid scintillation spectrometry to determine the recovery of [U-^{14}C]AMP or [8-^{3}H]cyclic AMP and this value was used to normalize each analysis to the original cell count.

In experiments utilizing radiolabeled precursors, the effluent of the liquid chromatograph was collected at 1-min intervals and these fractions were monitored by liquid scintillation spectrometry for their content of radioactivity.

Incubation of L5178Y cells with [^{3}H]ribavirin and extraction of RNA. Two separate experiments were carried out to determine whether [^{3}H]ribavirin is incorporated into cellular RNA.

In the first experiment, 2.5×10^8 L5178Y cells were suspended/50 ml of RPMI 1640 medium [supplemented with 4.0 mM L-glutamine, 2 mg/ml of sodium bicarbonate and 10% (v/v) dialyzed horse serum] and incubated in screw-capped 75 cm^2 tissue culture flasks (Falcon Plastics) for 3 hr at 37° either with 250 μM [^{3}H]ribavirin (sp. act. 4.0 mCi/m-mole), with or without added guanosine (100 μM), or with 44 nM [5-^{3}H]uridine (sp. act. 26 Ci/m-mole). Incubations were terminated by harvesting the cells by centrifugation and RNA was then extracted from the cells with a heated mixture of phenol, water and *m*-cresol, essentially as described by Wilkinson and Pitot[26]. Approximately 30 $A_{257\,nm}$ units of RNA were recovered from each sample of 2.5×10^8 cells.

In the second experiment, 1.0×10^8 L5178Y cells were removed from their culture flask during the mid-log phase of growth, diluted to 100 ml with fresh, warmed Fischer's medium and placed in a 500-ml screw-capped tissue culture flask. The cells were supplemented with 50 μM [^{3}H]ribavirin (sp. act. 100 mCi/m-mole) and 100 μM guanosine and the flask was flushed with CO_2, capped and incubated for 3 hr at 37°. The cells were then harvested and the RNA was extracted as above.

Electrophoretic analysis of RNA. The RNA preparations were fractionated on 8.0% (2.5% cross-linked) polyacrylamide gels (10 cm long) for 90 min at 5 mA. Under these conditions, ATP migrated through approximately 90 per cent of the gel. Typically, 4 $A_{257\,nm}$ units of RNA were loaded onto each gel. After electrophoresis, the gels were scanned at 257 nm (with a Gilford model 2520F Linear Transport accessory attached to a Gilford model 250 spectrophotometer) and then cut into 1.5-mm slices. The gel slices were digested overnight with 0.5 ml of Protosol–water (9:1, v/v), mixed with 3.5 ml Econofluor and monitored by liquid scintillation spectrometry with a counting efficiency of 24 per cent. The gels were calibrated by electrophoresis of authentic samples of transfer RNA and ATP.

Enzymatic peak-shift of analog ribonucleoside 5'-triphosphates. For the hexokinase-catalyzed peak-shift of analog ribonucleoside 5'-triphosphates, 28 units (20 μl) of hexokinase suspension was added to 40 μl of neutralized acid-soluble cell extract supplemented with 12.5 mM magnesium chloride and 0.125 M D-glucose and this mixture was incubated for 30 min at 37°; after acidification (with 10 μl of 2.5 M HCl), centrifugation (to remove precipitated protein), and neutralization (with 10 μl of 2.5 M NaOH), 15 μl of this solution was injected into the high-pressure liquid chromatograph for analysis. For the venom phosphodiesterase-catalyzed peak-shift experiment, 0.04 unit (25 μl) of phosphodiesterase was mixed with 5 μl of 1.0 M Tris–HCl (pH 8.9) containing 1.0 mM magnesium chloride and with 5 μl of neutralized acid-soluble cell extract and this mixture was incubated for 60 min at 37°; this solution was then deproteinized and analyzed by high-performance liquid chromatography.

RESULTS AND DISCUSSION

Evidence for metabolic formation of 5'-triphosphates of AICA ribonucleoside and its structural analogs. In-

itial metabolic studies of this group of compounds consisted of overnight incubation of fresh, heparinized human whole blood (2.0 ml) with each agent (0.5 mM), as described under Materials and Methods. Acid-soluble extracts of these blood samples were then prepared and analyzed by high-performance anion-exchange chromatography for evidence of nucleotide formation.

Incubation of human blood with AICA ribonucleoside, FICA ribonucleoside, pyrazofurin and ribavirin resulted in the appearance of unique new peaks in the di- and triphosphate regions of the high-performance liquid chromatograms (Fig. 2). The metabolites which were eluted later than ATP were adjudged to be the 5′-triphosphates of the various AICA-related compounds on the bases of three criteria. First, these metabolites were eluted in the triphosphate region of the chromatograms. Second, the material present in each of these putative triphosphate peaks was collected as it was eluted from the liquid chromatograph

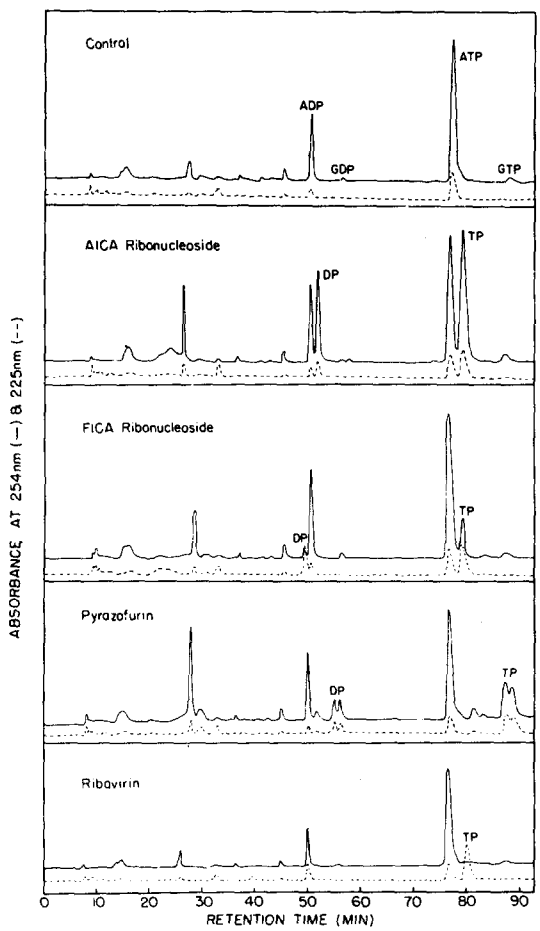

Fig. 2. High-performance liquid chromatograms of extracts prepared from human blood which had been incubated overnight with saline (control) or the specified agent (0.5 mM). The column effluent was monitored both at 254 nm (solid line, full-scale absorbance of 0.64) and at 225 nm (broken line, full-scale absorbance of 2.0). DP and TP designate the 5′-di- and -triphosphates, respectively, of the analog precursor specified in each chromatogram. Other conditions were as described in Materials and Methods.

and was found to exhibit the same u.v. spectral characteristics as the corresponding ribonucleoside drug precursors. Third, enzymatic peak-shift experiments with each of these extracts with either yeast hexokinase plus glucose or venom phosphodiesterase resulted (with the exception of the extract containing metabolites of pyrazofurin; see below) in the almost complete shift of the putative analog 5′-triphosphate peaks to the di- or monophosphate regions, respectively, of these chromatograms. In general, new peaks of metabolites of these agents were not readily discernible in the monophosphate region of the chromatograms shown in Fig. 2.

Pyrazofurin was unique among these agents in that it yielded twin peaks of di- and triphosphate metabolites, one of which was co-eluted with either GDP or GTP. Pyrazofurin has been reported to undergo facile interconversion between its α- and β-anomers [1], and these twin metabolite peaks are attributed to the presence of these two anomeric forms of each nucleotide species. This conclusion is supported by the finding that hexokanase and phosphodiesterase acted only on the early-eluting 5′-triphosphate metabolite, indicating that this species is the β-anomer and that the late-eluting species is the α-anomer. Presumably, the β-anomeric form of the drug was first metabolized to each nucleotide species and the α-anomers of these nucleotides were formed subsequent to these enzymatic events. It is not known whether the putative α-anomeric forms of these pyrazofurin nucleotides were actually formed intracellularly or whether they were generated during the acid-extraction of the cells.

Earlier reports that exogenously supplied AICA ribonucleoside is metabolized to IMP in the erythrocytes of some human donors [12, 13] and that inosine and hypoxanthine are metabolized to ITP in erythrocytes [27, 28] prompted further work to rule out the possibility that the triphosphate metabolite of AICA ribonucleoside might actually be ITP. The triphosphate metabolite of AICA ribonucleoside (λ_{max} = 267 nm in potassium phosphate buffer, pH 3.5) was clearly distinguishable spectrophotometrically from ITP (λ_{max} = 248 nm). Moreover, when a cell extract containing the triphosphate metabolite of AICA ribonucleoside was mixed with authentic ITP and injected into the liquid chromatograph, ITP was well-separated from AICA ribonucleoside 5′-triphosphate and was eluted between the latter and GTP. The addition of 10 mM sodium formate to an overnight incubation of blood with 1.0 mM AICA ribonucleoside resulted in the formation of little, if any, IMP or ITP.

AICA and FICA, the aglycones of AICA ribonucleoside and FICA ribonucleoside, respectively, were also metabolized extensively to their corresponding ribonucleoside 5′-triphosphates during overnight incubation with human whole blood. By contrast, 5-aminoimidazole-4-thiocarboxamide (the aglycone of thioAICA ribonucleoside) and 3-aminopyrazole-4-thiocarboxamide were metabolized very slowly but nonetheless detectably to their ribonucleoside 5′-triphosphates. 3-Aminopyrazole-4-carboxamide and 5-aminoimidazole-4-carboxhydrazide appeared to be metabolized to their ribonucleoside 5′-monophosphates, but no peaks of corresponding 5′-triphosphate

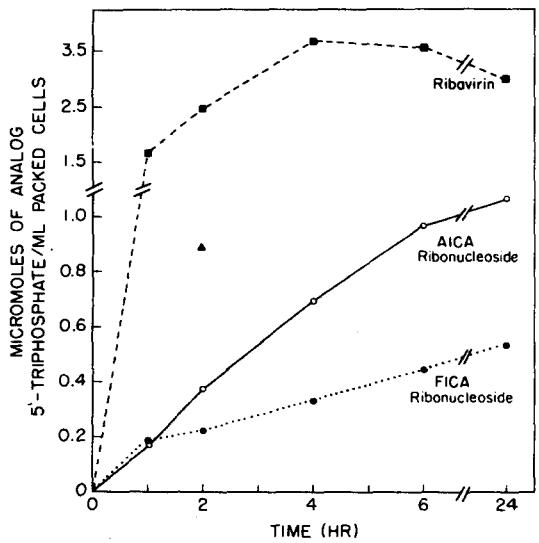

Fig. 3. Time-course of formation of 5'-triphosphates of AICA ribonucleoside, FICA ribonucleoside and ribavirin in whole human blood. Each blood incubation contained 1.0 mM analog ribonucleoside. These data were determined by high-performance liquid chromatographic analysis of the neutralized acid-soluble extract prepared from each blood incubation. The solid triangle represents the ATP level found in blood which had been incubated for 2 hr without drugs. Other details were as described in Materials and Methods.

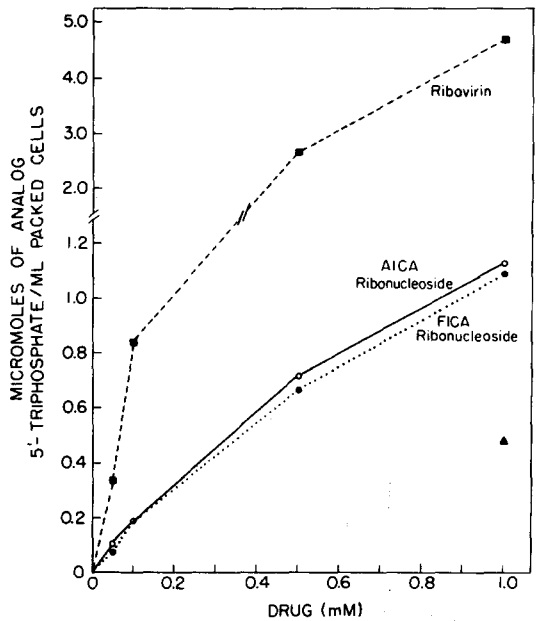

Fig. 4. Dose-dependence of analog 5'-triphosphate formation during a 4-hr incubation of whole human blood with AICA ribonucleoside, FICA ribonucleoside and ribavirin. The triphosphate levels were calculated from u.v. absorbance profiles of extracts fractionated by high-performance liquid chromatography. The solid triangle represents the ATP level found in blood which had been incubated for 4 hr without drugs. Other details were as described in Materials and Methods.

were discernible in the chromatograms; lack of evidence for the formation of the ribonucleoside 5'-triphosphates of the latter two agents may be due to co-chromatography of these analog nucleotides with either ATP or GTP. [2-^{14}C]imidazole, [5-^{14}C]3-amino-1,2,4-triazole, 5-aminoimidazole-1,2,3-triazole-4-carboxamide and 5-aminoimidazole-4-thiocarboxylic acid methyl ester were not detectably metabolized to nucleotide derivatives by human blood.

Time- and dose-dependence of formation of 5'-triphosphate metabolites in blood. A kinetic study of the formation of analog 5'-triphosphates from 1.0 mM AICA ribonucleoside, FICA ribonucleoside and ribavirin was carried out over a 24-hr incubation period with human blood cells (Fig. 3). Of these three agents, ribavirin was metabolized most rapidly to its 5'-triphosphate. Within 1 hr of incubation with 1.0 mM ribavirin, more than 50 per cent of the total drug available had been taken up and metabolized to its 5'-triphosphate. In this experiment, the cellular content of AICA ribonucleoside 5'-triphosphate and ribavirin 5'-triphosphate equaled or exceeded that of ATP.

Figure 4 presents the dose-dependence of 5'-triphosphate formation during 4-hr incubations of blood with AICA ribonucleoside, FICA ribonucleoside and ribavirin. Triphosphate formation was demonstrable with all three agents at concentrations as low as 50 μM.

Evidence for cellular metabolism of AICA ribonucleoside and its structural analogs via a nucleoside kinase. Although pyrazofurin, a C-nucleoside, must of necessity be converted to its 5'-monophosphate via a nucleoside kinase, AICA ribonucleoside has been reported not to be a substrate for adenosine kinase purified from human tumor cells [17]. While AICA can be metabolized to its ribonucleoside 5'-monophosphate via adenine phosphoribosyltransferase [18–20], the substrate activity of AICA ribonucleoside with purine nucleoside phosphorylase appears to vary greatly among different tissues [14, 15] and has not yet, to the authors' knowledge, been determined with the enzyme from human erythrocytes. Therefore, it was of interest to learn by which of the two possible pathways—direct phosphorylation or phosphorolysis followed by phosphoribosylation—these AICA ribonucleosides are metabolized to their corresponding 5'-monophosphates by intact human blood cells. This was studied experimentally by comparing the incorporation of radioactivity from [U-^{14}C]D-glucose into the analog ribonucleoside 5'-triphosphates formed metabolically from AICA, AICA ribonucleoside, FICA, FICA ribonucleoside and ribavirin.

The analog ribonucleoside 5'-triphosphates formed during a 3-hr incubation of human blood cells with AICA or FICA plus [U-^{14}C]D-glucose were found to contain high levels of radioactivity (Table 1). The specific activities of the ribonucleoside 5'-triphosphates formed from these two aglycones were 80–90 per cent that of the [U-^{14}C]D-glucose supplied to the cells;* thus, the exogenous [U-^{14}C]D-glucose sup-

* Although the specific activity of the [U-^{14}C]D-glucose employed in this experiment was 940 des./min/nmole, only five out of the six carbon atoms of each glucose molecule are retained in ribose-containing compounds formed metabolically therefrom.

Table 1. Incorporation of radioactivity from [U-^{14}C]D-glucose into analog ribonucleoside 5'-triphosphates formed metabolically in human blood cells *in vitro**

Analog precursor	Analog ribonucleoside 5'-triphosphate (nmoles formed/2.0 ml of blood)	Specific activity of analog ribonucleoside 5'-triphosphate (dis./min/nmole)
AICA	147	617
AICA ribonucleoside	137	0
FICA	144	691
FICA ribonucleoside	83	237
Ribavirin	486	63

* Two-ml samples of fresh, heparinized, human whole blood were placed in each of six tubes and the blood cells were washed two times with cold PBS and resuspended in cold PBS to the original hematocrit. These cell suspensions were then incubated at 37° for 15 min, after which time each tube received: 250 μl of a solution containing 130 μmoles potassium phosphate (*p*H 7.4), 1.2 mg each of penicillin G and streptomycin sulfate and 20 μmoles [U-^{14}C]D-glucose (sp. act. 0.42 mCi/m-mole); and 320 μl of either saline or analog precursor (0.64 μmole of AICA ribonucleoside, FICA ribonucleoside or ribavirin, or 2.57 μmoles of either AICA or FICA). Incubation at 37° was then continued for 3 hr. Incubations were terminated by diluting the cell suspensions with 10 ml of cold physiological saline, collecting the cells by centrifugation and extracting each cellular sample with 10 ml of cold 0.5 M perchloric acid. Extracts were clarified, neutralized with KOH and concentrated to a final volume of 200 μl. A portion (5 μl) of each extract was analyzed by high-performance liquid chromatography; 1-min fractions of the column effluent were collected and monitored for radioactivity by liquid scintillation spectrometry.

plied most of the phosphorylated ribose intermediates used by the blood cells to metabolize AICA and FICA to their corresponding ribonucleotides. By contrast, the 5'-triphosphate formed from AICA ribonucleoside did not incorporate any detectable radioactivity from the [U-^{14}C]D-glucose. This result suggests very strongly that the ribose moiety of AICA ribonucleoside was not cleaved from this compound during its metabolism to AICA ribonucleoside 5'- monophosphate and that this metabolism, therefore, occurred via the action of a nucleoside kinase. Ribavirin 5'-triphosphate was found to contain a relatively low level of radioactivity derived from the [U-^{14}C]D-glucose; from the specific activity of this latter 5'-triphosphate, it can be estimated that ribavirin is cleaved only slightly (<10 per cent) during metabolism to its 5'-monophosphate and that ribavirin, like AICA ribonucleoside, is metabolized predominately by a nucleoside kinase. The specific activity of the 5'-triphosphate formed from FICA ribonucleoside was intermediate between that found for the aglycones (AICA and FICA) and that found for the other ribonucleosides (AICA ribonucleoside and ribavirin); this latter result indicates that FICA ribonucleoside was also metabolized chiefly via a nucleoside kinase but that a significant portion (approximately one-third) of the FICA ribonucleoside underwent phosphorolysis prior to being metabolized to its 5'-monophosphate. Upon metabolism of FICA ribonucleoside to its aglycone, the latter may have incorporated radioactive ribose either from ribose-1-phosphate via purine nucleoside phosphorylase or from 5-phospho-α-D-ribose-1-pyrophosphate via a phosphoribosyltransferase-mediated reaction. From the above results it is apparent that AICA ribonucleoside and its structural analogs are not phosphorolyzed to any great extent by human erythrocytes, even though these cells contain high levels of purine nucleoside phosphorylase [16].

The above evidence that AICA ribonucleoside, FICA ribonucleoside and ribavirin are metabolized to their 5'-monophosphates chiefly via a nucleoside

Table 2. Pool sizes of ribonucleoside 5'-triphosphates in L5178Y cells after 1-hr treatment with ribavirin*

Conc of ribavirin (μM)	CTP	UTP	ATP	GTP	Ribavirin 5'-triphosphate
		(pmoles nucleotide/10^6 cells)			
0	172 ± 8	398 ± 43	1561 ± 132	294 ± 25	
100	214 ± 37	574 ± 53	1819 ± 178	142 ± 8†	103 ± 29
500	259 ± 28	684 ± 48†	1874 ± 236	125 ± 24†	473 ± 3

* L5178Y cells were harvested from suspension culture by centrifugation and were resuspended in RPMI 1640 medium (supplemented with 4.0 mM L-glutamine and 10% dialyzed horse serum) to a cell density of 8.3 × 10^6 cells/ml. Ten-ml portions of this cell suspension were supplemented with the specified concentration of ribavirin and were incubated for 60 min at 37°. These incubations were terminated by pelleting the cells (121 *g* for 10 min), resuspending the cell pellets briefly in 500 μl of cold water and immediately extracting the cells by the rapid addition of 5.0 ml of cold 1.0 M perchloric acid containing 4 × 10^5 dis./min of [8-^{3}H]cyclic AMP. These cell extracts were clarified, neutralized with KOH and concentrated to a final volume of 200 μl. A portion (15 μl) of each extract was analyzed by high-performance liquid chromatography. The experiments were performed in duplicate and the results are expressed as the mean ± the average deviation of the two analyses.

† Indicates statistical significance at $P < 0.05$ or greater, using a two-tailed Student's *t*-test.

kinase is supported by the recent finding that these compounds are reasonably good substrates for adenosine kinase purified from rabbit liver.*

Metabolism of ribavirin in L5178Y cells. The facile metabolism of compounds of this general structural class to their corresponding 5'-triphosphates in human blood cells (predominately erythrocytes) raised the question as to whether these analog triphosphates might be incorporated into RNA in nucleated cells. This question was addressed by studying the metabolism of ribavirin in L5178Y cells.

In an initial experiment, L5178Y cells were incubated with unlabeled ribavirin (100 or 500 μM) for 1 hr and the resultant acid-soluble nucleotides were analyzed by high-performance anion-exchange chromatography. As summarized in Table 2, ribavirin 5'-triphosphate was formed in the L5178Y cells in substantial amounts within 1 hr, in a dose-dependent manner. This result was subsequently verified using [^{3}H]ribavirin. The pool size of GTP in the L5178Y cells was depressed significantly by the 1-hr treatment with 100 μM ribavirin. This finding is consistent with reports that ribavirin 5'-monophosphate potently inhibits inosinate dehydrogenase and thereby inhibits the *de novo* biosynthesis of guanine nucleotides [2, 29, 30]. Quite unexpectedly, the cellular pools of CTP and UTP were increased substantially by treatment of the cells with ribavirin. Very recently, Lowe *et al.* [31] have reported very similar effects of ribavirin on the pool sizes of ribonucleoside 5'-triphosphates in L5178Y cells.

In a subsequent experiment, L5178Y cells were incubated with 250 μM [^{3}H]ribavirin (sp. act. 4.0 mCi/m-mole), both in the absence and presence of 100 μM guanosine, for 3 hr and the RNA was then extracted from these cells and analyzed for incorporation of radioactivity. The [^{3}H]ribavirin was not incorporated at detectable levels into the RNA of the L5178Y cells.† In a parallel incubation, these L5178Y cells were shown to readily incorporate [^{3}H]uridine into their RNA, thereby verifying the competency of these cells to synthesize RNA under the experimental conditions employed. In a similar manner, incubation of L5178Y cells with 50 μM [^{3}H]ribavirin (sp. act. 100 mCi/m-mole) plus 100 μM guanosine for 3 hr did not result in the incorporation of detectable amounts of radioactivity into RNA. It has been estimated that the conditions of this latter experiment would have allowed detection of the incorporation of 1 mole of [^{3}H]ribavirin/14,000 moles of total nucleoside residues present in the RNA. Ribavirin is known to be inhibitory to the growth of L5178Y cells [31].

Recognition of the rapid and extensive metabolism of agents such as FICA ribonucleoside, pyrazofurin and ribavirin to their corresponding 5'-polyphosphates provides new alternatives to consider concerning the mechanism of action of these interesting compounds. Obviously, much speculation is possible concerning the many potential biochemical activities of these analog polyphosphates.

Small amounts (approximately 1 mg/day) of AICA are excreted in the urine of normal humans [32, 33]. The urinary level of AICA is elevated in a number of pathological conditions, including vitamin B_{12} deficiency [34, 35], folic acid deficiency [34, 35], liver diseases [34], the Lesch–Nyhan syndrome [36] and leukemia [37, 38]. In addition, methotrexate has been shown to cause an elevated excretion of AICA [34, 37]. To the extent that this elevated output of AICA is limited to a few tissues, it is possible that local tissue levels of AICA ribonucleoside 5'-monophosphate could increase sufficiently to allow the accumulation of AICA ribonucleoside 5'-triphosphate within such tissues.

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* R. L. Miller and D. L. Adamczyk, personal communication.

† Small amounts of radioactivity were found to be present in all RNA samples extracted from [^{3}H]ribavirin-treated L5178Y cells. However, electrophoretic fractionation of these RNA preparations established that this radioactive material was of a very low molecular weight, co-migrating with added ATP, and that no radioactivity was apparent anywhere else in the gels.

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