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IMPROVED METHODS FOR THE STUDY OF DRUG EFFECTS ON PURINE METABOLISM AND THEIR APPLICATION TO NEBULARINE AND 7-DEAZANEBULARINE*

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Abstract—Procedures are described by which the apparent rates of 23 enzymes of purine metabolism can be calculated from data obtained using intact Ehrlich ascites cells incubated *in vitro*, and these have been applied to the study of the effects of certain purine analogs and derivatives. Nebularine (9-β-D-ribofuranosyl purine), 7-deazanebularine (7-β-D-ribofuranosyl pyrrolopyrimidine) and related compounds inhibit nucleotide synthesis from radioactive adenine, guanine and hypoxanthine in Ehrlich ascites tumor cells *in vitro*, and also inhibit phosphoribosyl pyrophosphate accumulation in cells incubated with glucose. Nebularine and 7-deazanebularine inhibit the growth of cultured leukemia RPMI 6410 cells.

SNYDER *et al.*[1] have described a procedure by which the apparent activities of eight enzymes of purine ribonucleotide synthesis and interconversion could be calculated when Ehrlich ascites tumor cells were incubated *in vitro* with hypoxanthine-^{14}C. The possible application of this procedure to measuring drug effects on these enzyme activities was also described. In the present study this procedure is extended in two ways: (1) apparent activities of enzymes of purine nucleotide, nucleoside and base catabolism are calculated; and (2) enzymes measured when cells are incubated with radioactive adenine and guanine are included. These methods have been applied to the study of the effects of certain purine nucleoside analogs and derivatives, especially nebularine and 7-deazanebularine.

Nebularine (9-β-D-ribofuranosyl purine; purine ribonucleoside) (Fig. 1) is a highly toxic natural product,[2–4] which is enzymatically phosphorylated to mono-, di- and triphosphate derivatives,[5–9] but whose biochemical effects have never been defined. 7-Deazanebularine (7-β-D-ribofuranosyl pyrrolo[2,3-*d*]pyrimidine) (Fig. 1), a synthetic compound,[10] has recently also been shown to be cytotoxic and to be phosphorylated to mono-, di- and triphosphorylated derivatives and incorporated into both RNA and DNA;[11] the coding properties of oligo- and polynucleotides containing 7-deazanebularine have been studied.[12,13] Numerous other natural and synthetic ribofuranosyl pyrrolopyrimidines have been tested for growth inhibitory activity, and the effects of a few—especially tubercidin, toyocamycin and sangivamycin—on

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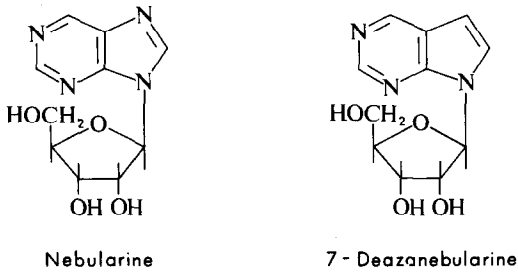


FIG. 1. Structures of nebularine and 7-deazanebularine.

nucleic acid metabolism have been studied.[3,4] Except for inhibition of purine biosynthesis *de novo* by tubercidin,[14–16] effects of ribofuranosyl pyrrolopyrimidines on acid-soluble purine nucleotide metabolism have not been reported.

This paper reports studies of the effects of nebularine, 7-deazanebularine and a number of related ribofuranosyl pyrrolopyrimidines and purine ribonucleosides on purine ribonucleotide syntheses and metabolism.

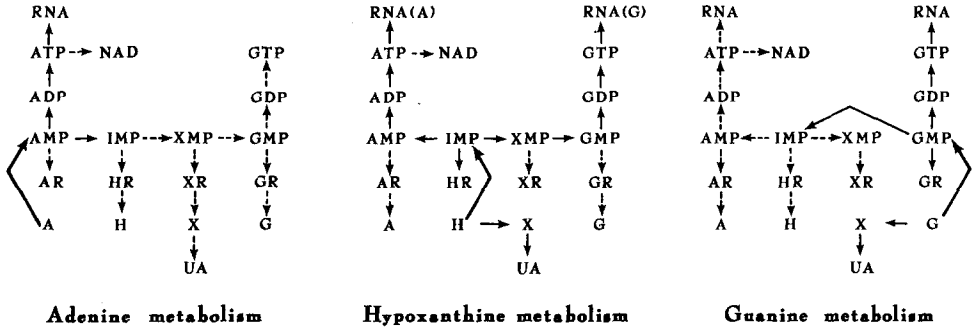
MATERIALS AND METHODS

The following compounds were provided by the Drug Evaluation Branch, Drug Research and Development, National Cancer Institute, Bethesda, Md.: 7-deazanebularine (NSC 107519); nebularine (NSC 65423); 4-amino-7-β-D-ribofuranosyl pyrrolo(2,3-*d*)pyrimidine (NSC 56408); 6-hydrazino-9-β-D-ribofuranosyl purine (NSC 29408); 4-hydrazino-7-β-D-ribofuranosyl pyrrolo(2,3-*d*)-pyrimidine (NSC 111360); 6-methylamino-9-β-D-ribofuranosyl purine (NSC 29409); 4-methylamino-7-β-D-ribofuranosyl pyrrolo(2,3-*d*)pyrimidine (NSC 103791); 4-dimethylamino-7-β-D-ribofuranosyl pyrrolo(2,3-*d*)pyrimidine (NSC 100279); 4-oxo-7-β-D-ribofuranosyl pyrrolo(2,3-*d*)pyrimidine (NSC 99439); 6-methoxy-9-β-D-ribofuranosyl purine (NSC 30606); 4-methoxy-7-β-D-ribofuranosyl pyrrolo(2,3-*d*)pyrimidine (NSC 101160); 6-mercapto-9-β-D-ribofuranosyl purine (NSC 4911); 4-mercapto-7-β-D-ribofuranosyl pyrrolo(2,3-*d*)pyrimidine (NSC 100278); 4-amino-7-β-D-(3′-deoxyribofuranosyl) pyrrolo(2,3-*d*)pyrimidine (NSC 124154); 4-amino-5-chloro-7-β-D-ribofuranosyl pyrrolo(2,3-*d*)pyrimidine (NSC 124149); 4-amino-7-β-D-ribofuranosyl pyrrolo(2,3-*d*)pyrimidine 5-carboxamidine (NSC 131663); 4-chloro-7-β-D-ribofuranosyl pyrrolo(2,3-*d*) pyrimidine 5-carbonitrile (NSC 117839); 5-bromo-4-methylthio-7-β-D-ribofuranosyl pyrrolo(2,3-*d*)-pyrimidine (NSC 113942); 4-dimethylformamidino-7-β-D-ribofuranosyl pyrrolo(2,3-*d*)pyrimidine 5-carbonitrile (NSC 143686); 4-amino-6-methylthio-7-β-D-ribofuranosyl pyrrolo(2,3-*d*)pyrimidine 5-carboxamide (NSC 117838); 7-β-D-ribofuranosyl pyrrolo (2,3-*d*)pyrimidine 5-thiocarboxamide (NSC 143687); 4-amino-6-bromo-7-β-D-ribofuranosyl pyrrolo(2,3-*d*)pyrimidine 5-carbonitrile (NSC 113951); 4-amino-6-bromo-7-β-D-ribofuranosyl pyrrolo(2,3-*d*)pyrimidine 5-carboxamide (NSC 113943); 4-amino-5-iodo-7-β-D-ribofuranosyl pyrrolo(2,3-*d*)pyrimidine (NSC 113939); 4-mercapto-7-β-D-ribofuranosyl pyrrolo(2,3-*d*)pyrimidine 5-carbonitrile (NSC 116097); 4-methylthio-7-β-D-tibofuranosyl pyrrolo(2,3-*d*)pyrimidine 5-carbonitrile (NSC 116098); 4-methylthio-7-β-D-ribofuranosyl pyrrolo(2,3-*d*)pyrimidine (NSC 105826); 4,5-dichloro-7-β-D-ribofuranosyl pyrrolo(2,3-*d*)pyrimidine (NSC

124143); and 4-chloro-5-iodo-7-β-D-ribofuranosyl pyrrolo(2,3-*d*)pyrimidine (NSC 113940).

Procedures for the maintenance, preparation and incubation of Ehrlich ascites tumor cells have been described previously, as have methods for the measurement of radioactivity in individual purine bases, ribonucleosides, ribonucleotides and nucleic acids,[17–19] for the measurement of PP-ribose-P[20] and for the chromatography of acid soluble nucleotides.[21] Results presented are averages of duplicate analyses in two experiments. Human leukemia RPMI* 6410 cells (obtained from Associated Biomedic Systems) were grown in RPMI 1640 medium containing 10% fetal calf serum, 100 μg/ml of streptomycin and 100 units/ml of penicillin.

A method for evaluating the effects of drugs on apparent activities of several individual enzymes of hypoxanthine-^{14}C metabolism has been reported.[1] Here it is extended to other reactions of hypoxanthine-^{14}C metabolism and to reactions measured when cells are incubated with radioactive adenine or guanine.



SCHEME 1. Enzymatic reactions of adenine, hypoxanthine and guanine metabolism.

The reactions studied with each radioactive purine base are shown in Scheme 1. The apparent activity of each enzymatic reaction shown there is presented in terms of the amount of radioactive substrate metabolized by that enzyme; Table 1 gives the arithmetic sums denoting each reaction. Other studies[19] have indicated that under the conditions used here radioisotope flow from purine bases to ATP and GTP is virtually equivalent to the observed change in chemical composition of the triphosphate pools.

Table 1 also gives typical control values for the apparent activity of each enzymatic reaction measured. Because of the low values found in some compounds, the significance of measurements of certain reactions is not great; these reactions are indicated by a footnote (‡) in Table 1 and by dotted lines in Scheme 1, and they are usually not considered in studies of drug action.

By comparison of data from control and drug-treated cells, per cent inhibition of each enzyme reaction can be calculated as previously described;[1] corrections are made for inhibition of reactions prior to that under consideration. The computer programs which perform these calculations are written in APL/360, and may be obtained from the authors.

* RPMI = Roswell Park Memorial Institute.

TABLE 1. SUMMATIONS REPRESENTING APPARENT ACTIVITIES OF ENZYMATIC PROCESSES OF RADIOACTIVE ADENINE, HYPOXANTHINE AND GUANINE METABOLISM*

Reaction†	Summation
Adenine metabolism	
A phosphoribosyl-transferase	AMP + ADP + ATP + NAD + NA + IMP + XMP + GMP + GDP + GTP + AR + H + HR + X + XR + G + GR + UA (27,532)
AMP kinase	ADP + ATP + NAD + NA (25,385)
ADP kinase	ATP + NAD + NA (24,620)
RNA polymerase	NA (531)
AMP deaminase	IMP + XMP + GMP + GDP + GTP + H + HR + X + XR + G + GR + UA (2013)
IMP dehydrogenase‡	XMP + GMP + GDP + GTP + X + XR + G + GR + UA (1222)
GMP synthetase‡	GMP + GDP + GTP + G + GR (1153)
GMP kinase‡	GDP + GTP (1112)
GDP kinase‡	GTP (182)
AMP dephosphorylase‡	AR (8)
IMP dephosphorylase‡	H + HR (654)
XMP dephosphorylase‡	X + XR + UA (68)
GMP dephosphorylase‡	G + GR (12)
HR phosphorylase‡	H (527)
XR phosphorylase‡	X + UA (67)
GR phosphorylase‡	G (11)
Xanthine oxidase‡	UA (49)
Hypoxanthine metabolism	
H phosphoribosyl-transferase	IMP + AMP + ADP + ATP + NAD + XMP + GMP + GDP + GTP + A + AR + HR + X + XR + G + GR + NA (18,261)
AMPS synth. + lyase	AMP + ADP + ATP + NAD + NA-A + A + AR (14,850)
AMP kinase	ADP + ATP + NAD + NA-A (14,671)
ADP kinase	ATP + NAD + A NA (13,800)
IMP dehydrogenase	XMP + GMP + GDP + GTP + XR + G + GR + G + NA-G (1802)
GMP synthetase	GMP + GDP + GTP + G + GR + NA-G (1748)
GMP kinase	GDP + GTP + NA-G (1632)
GDP kinase	GTP + NA-G (1235)
RNA polymerase	NA-A + NA-G
AMP dephosphorylase‡	A + AR (47)
IMP dephosphorylase	HR (902)
XMP dephosphorylase‡	XR (50)
GMP dephosphorylase‡	G + GR (22)
AR phosphorylase‡	A (39)
GR phosphorylase‡	G (21)
Xanthine oxidase	X + UA (734)
Guanine metabolism	
G phosphoribosyl-transferase	GMP + GDP + GTP + NA + IMP + XMP + AMP + ADP + ATP + NAD + A + AR + H + HR + XR + GR (14,792)
GMP kinase	GDP + GTP + NA (13,086)
GDP kinase	GTP + NA (12,704)
RNA polymerase	NA (665)
GMP reductase	IMP + XMP + AMP + ADP + ATP + NAD + A + AR + H + HR + XR (1111)
IMP dehydrogenase‡	XMP + XR (58)
AMPS synth. + lyase‡	AMP + ADP + ATP + NAD + A + AR (564)
AMP kinase*	ADP + ATP + NAD (157)

TABLE 1—*continued*

Reaction†	Summation
ADP kinase‡	ATP + NAD (69)
AMP dephosphorylase‡	A + AR (7)
IMP dephosphorylase‡	H + HR (18)
XMP dephosphorylase‡	XR (45)
GMP dephosphorylase	GR (150)
AR phosphorylase‡	A (1)
HR phosphorylase‡	H (5)
G deaminase	X + UA (7194)
Xanthine oxidase	UA (331)

* Abbreviations used: NA, nucleic acids; A, adenine; AR, adenosine; H, hypoxanthine; HR, inosine; X, xanthine; XR, xanthosine; UA, uric acid; G, guanine; GR, guanosine, NA-A, nucleic acid adenine; NA-G, nucleic acid guanine; AMPS, adenylosuccinate. Typical control values, in cpm actually measured, are given in parentheses for each reaction.

† Enzyme names: A phosphoribosyltransferase, adenylate: pyrophosphate phosphoribosyltransferase (EC 2.4.2.7); H phosphoribosyltransferase, inosinate: pyrophosphate phosphoribosyltransferase (EC 2.4.2.8) measured using hypoxanthine-^{14}C; G phosphoribosyltransferase, inosinate: pyrophosphate phosphoribosyltransferase (EC 2.4.2.8) measured using guanine-^{14}C; AMP deaminase, adenylate aninohydrolase (EC 3.5.4.6); IMP dehydrogenase, inosinate: NAD oxidoreductase (EC 1.2.1.14); GMP synthetase, xanthylate ligase (AMP) (EC 6.3.4.1); AMPS synthetase + lyase, inosinate: L-aspartate ligase (GDP) (EC 6.3.4.4) plus adenylosuccinate: AMP-lyase (EC 4.3.2.2); RNA polymerase, nucleoside triphosphate: RNA nucleotidyltransferase (EC 2.7.7.6); GMP reductase, reduced NADP: GMP oxidoreductase (deaminating) (EC 1.6.6.8); xanthine oxidase, xanthine: oxygen oxidoreductase (EC 1.3.2.3); guanine deaminase, guanine aminohydrolase (EC 3.5.4.3) and AR, HR, XR and GR phosphorylases, (probably) purinenucleoside: orthophosphate ribosyltransferase (EC 2.4.2.1). The following reactions may either be catalyzed by more than one enzyme, or the identity of the enzyme(s) involved in these cells is not certain; it is the process rather than an individual enzyme that is listed above: AMP kinase, ADP kinase, GMP kinase, GDP kinase, AMP dephosphorylase, IMP dephosphorylase, XMP dephosphorylase, GMP dephosphorylase.

‡ The apparent activity of this action is not considered significant because of the low amount of radioactivity measured. This reaction is indicated by a dotted line in Scheme 1.

RESULTS

Table 2 shows the effects of nebularine and 7-deazanebularine on the apparent activities of a number of enzymes of purine ribonucleotide synthesis and metabolism in Ehrlich ascites tumor cells *in vitro*. Nebularine and 7-deazanebularine, 100 μM both produced greater than 50 per cent inhibition of ribonucleotide synthesis from radioactive adenine, hypoxanthine and guanine, and 7-deazanebularine also inhibited the incorporation of GTP into RNA. The apparent stimulation of adenylate deaminase activity produced by these compounds will be considered further below. The apparent stimulation of xanthine oxidase and guanine deaminase activities probably is a secondary consequence of inhibition of nucleotide synthesis, so that radioactive purine base concentrations remain higher than in the controls. Other reactions were not significantly affected by either nebularine or 7-deazanebularine.

In attempts to gain information regarding structure–activity relationships in this system, a number of other ribofuranosyl pyrrolopyrimidines were tested (at a concentration of 1 mM) on hypoxanthine-^{14}C metabolism in Ehrlich ascites tumor cells *in vitro*. Some purine ribonucleosides analogous to specific ribofuranosyl pyrrolopyrimidines were also included, as were nebularine and 7-deazanebularine at this higher concentration. The predominant effect of most of these compounds was inhibition of ribonucleotide syntheses; these data are presented in Table 3. 4-Amino- and 4-

TABLE 2. EFFECTS OF NEBULARINE AND 7-DEAZANEBULARINE ON PURINE METABOLISM*

Precursor	Reaction	Apparent enzyme activity % Inhibition by nebularine (100 μM)	% Inhibition by 7-deazanebularine (50 μM)	(100 μM)
Adenine-^{14}C	Adenine phosphoribosyl-transferase	69·6	12·9	64·6
	AMP kinase	6·5	2·9	5·7
	ADP kinase	0·1	2·8	1·5
	RNA polymerase	†	†	6·1
	AMP deaminase	−177	−88	−172
Hypoxanthine-^{14}C	Hypoxanthine phospho-ribosyltransferase	72·9	30·9	61·9
	AMPS synthetase + lyase	44·9	8·0	21·5
	AMP kinase	3·7	0·1	0·2
	ADP kinase	−1·2	0·5	−1·4
	IMP dehydrogenase	−60·9	−0·4	8·8
	GMP synthetase	52·7	5·6	5·3
	GMP kinase	19·1	0·0	−0·6
	GDP kinase	−18·0	1·9	0·6
	Xanthine oxidase	53·5	−35·5	−54·8
Guanine-^{14}C	Guanine phosphoribosyl-transferase	59·4	†	52·9
	GMP kinase	12·4	†	15·1
	GDP kinase	−3·9	†	−3·9
	RNA polymerase	†	†	45·0
	GMP reductase	−140	†	†
	Guanine deaminase	−89·2	†	−118

* Ehrlich ascites tumor cells, 2 per cent by volume, were incubated for 20 min at 37° with shaking in an atmosphere of air in 0·1 ml Fischer's medium containing 25 mM sodium phosphate buffer, pH 7·4, and 5·5 mM glucose, with and without nebularine or 7-deazanebularine. Hypoxanthine-^{14}C, adenine-^{14}C or guanine-^{14}C was added to a final concentration of 50 μM, and incubation continued for 60 min. Control values are given in Table 1.

† Not measured.

hydrazino ribofuranosyl pyrrolopyrimidines were almost as active as 7-deazanebularine, whereas 4-methylamino, 4-dimethylamino and 4-methoxy ribofuranosyl pyrrolopyrimidines were less active; the 4-mercapto derivative was completely inactive. Nebularine, 6-hydrazino and 6-methylamino purine ribonucleosides had about the same activity as their ribofuranosyl pyrrolopyrimidine analogs, whereas 6-methoxy and 4-mercapto purine ribonucleosides were more active than the analogous ribofuranosyl pyrrolopyrimidines. Ribofuranosyl pyrrolopyrimidines substituted on the 5- and 6-positions were in general less active, although strong inhibition was produced by 4-amino ribofuranosyl pyrrolopyrimidine 5-carboxamidine.

Additional effects of these compounds are given in Tables 4 and 5 (only effects greater than 10 per cent inhibition are listed). In Table 4 are listed those ribofuranosyl pyrrolopyrimidines that apparently produced some inhibition of inosinate dehydrogenase activity. In general this inhibition was not potent, but in several cases it was equivalent to the inhibition of purine ribonucleotide synthesis reported in Table 3. Three compounds (4-methylthio ribofuranosyl pyrrolopyrimidine 5-carbonitrile,

TABLE 3. INHIBITION OF NUCLEOTIDE SYNTHESIS*

Base	Compound tested Substituent at position† 9/7	6/4	7/5	Nucleotide synthesis (% inhibition)
Purine	Ribosyl	H		91·9
Pyrrolopyrimidine	Ribosyl	H		96·2
Pyrrolopyrimidine	Ribosyl	NH_2		91·2
Pyrrolopyrimidine	3′-Deoxyribosyl	NH_2		91·4
Purine	Ribosyl	NHNH		88·8
Pyrrolopyrimidine	Ribosyl	NHNH		87·7
Purine	Ribosyl	$NH(CH_3)$		76·7
Pyrrolopyrimidine	Ribosyl	$NH(CH_3)$		69·0
Pyrrolopyrimidine	Ribosyl	$N(CH_3)_2$		56·0
Pyrrolopyrimidine	Ribosyl	OH		79·6
Purine	Ribosyl	OCH_3		86·7
Pyrrolopyrimidine	Ribosyl	OCH_3		38·8
Purine	Ribosyl	SH		62·1
Pyrrolopyrimidine	Ribosyl	SH		−8·9
Pyrrolopyrimidine	Ribosyl	H	$CSNH_2$	5·8
Pyrrolopyrimidine	Ribosyl	NH_2	Cl	50·5
Pyrrolopyrimidine	Ribosyl	NH_2	$HNCNH_2$	89·8
Pyrrolopyrimidine 6-$S(CH_3)$	Ribosyl	NH_2	$CONH_2$	15·9
Pyrrolopyrimidine 6-Br	Ribosyl	NH_2	$C\equiv N$	−1·8
Pyrrolopyrimidine 6-Br	Ribosyl	NH_2	$CONH_2$	−6·2
Pyrrolopyrimidine	Ribosyl	NH_2	I	−1·0
Pyrrolopyrimidine	Ribosyl	$N{=}CH-N(CH_3)_2$	$C\equiv N$	7·4
Pyrrolopyrimidine	Ribosyl	SH	$C\equiv N$	16·1
Pyrrolopyrimidine	Ribosyl	$S(CH_3)$	$C\equiv N$	67·4
Pyrrolopyrimidine	Ribosyl	$S(CH_3)$		55·2
Pyrrolopyrimidine	Ribosyl	$S(CH_3)$	Br	1·5
Pyrrolopyrimidine	Ribosyl	Cl	$C\equiv N$	27·6
Pyrrolopyrimidine	Ribosyl	Cl	Cl	59·4
Pyrrolopyrimidine	Ribosyl	Cl	I	50·8

* Tumor cells were incubated as described in Table 2 with 50 μM hypoxanthine-^{14}C with and without 1 mM test compound. Control nucleotide radioactivity: 17,420 cpm.

† Positions 9, 6 and 7 refer to the purine ring, and positions 7, 4 and 5 refer to the analogous positions on the pyrrolo(2,3-*d*)pyrimidine ring; position 6 in the latter ring is analogous to position 8 in the purine ring.

4-hydrazino ribofuranosyl pyrrolopyrimidine, and 6-hydrazino ribofuranosyl purine) inhibited the apparent activity of guanylate synthetase by about 20 per cent. In addition, the conversion of inosinate to adenylate was inhibited 28 per cent each by 4-hydrazino ribofuranosyl pyrrolopyrimidine and 6-hydrazino ribofuranosyl purine, and was inhibited 14 per cent by 6-methoxy ribofuranosyl purine.

Table 5 lists those compounds which are apparent inhibitors of one or more reactions of purine ribonucleotide phosphorylation in Ehrlich ascites tumor cells incubated *in vitro* with hypoxanthine-^{14}C. Only in two cases were inhibitions of approximately 50 per cent produced.

A possible basis for the inhibition of ribonucleotide synthesis from radioactive adenine, hypoxanthine and guanine reported in Tables 2 and 3 is the inhibition of PP-ribose-P synthesis; this compound is co-substrate of both adenine (EC 2.4.2.7)

TABLE 4. INHIBITION OF INOSINATE DEHYDROGENASE ACTIVITY*

Compounds tested	Apparent activity of inosinate dehydrogenase (% inhibition)
4-Methylthio ribofuranosyl pyrrolopyrimidine	53·9
4-Methoxy ribofuranosyl pyrrolopyrimidine	44·4
4-Dimethylamino ribofuranosyl pyrrolopyrimidine	39·0
4-Amino-6-bromo ribofuranosyl pyrrolopyrimidine 5-carboxamide	36·8
5-Bromo 4-methylthio ribofuranosyl pyrrolopyrimidine	25·9
4-Dimethylformamidino rirofuranosyl pyrrolopyrimidine 5-carbonitrile	25·9
4-Chloro ribofuranosyl pyrrolopyrimidine 5-carbonitrile	26·3
4-Amino-5-chloro ribofuranosyl pyrrolopyrimidine	22·4
4-Mercapto ribofuranosyl pyrrolopyrimidine 5-carbonitrile	17·9
4-Methylamino ribofuranosyl pyrrolopyrimidine	16·5
4-Amino-6-bromo ribofuranosyl pyrrolopyrimidine 5-carbonitrile	12·4

* Tumor cells were incubated as described in Table 2 with 50 μM hypoxanthine-^{14}C with and without 1 mM test compound. Control apparent activity: 1910 cpm.

TABLE 5. INHIBITION OF NUCLEOTIDE PHOSPHORYLATION*

Compounds tested	Apparent enzyme activity (% inhibition)
GMP kinase (1590)	
4-Hydrazino ribofuranosyl pyrrolopyrimidine	21·5
GDP kinase (1330)	
4-Chloro ribofuranosyl pyrrolopyrimidine 5-carbonitrile	56·9
4-Chloro-5-iodo ribofuranosyl pyrrolopyrimidine	47·1
4-Methylthio ribofuranosyl pyrrolopyrimidine 5-carboxamide	26·3
4,5-Dichloro ribofuranosyl pyrrolopyrimidine	20·7
Ribofuranosyl pyrrolopyrimidine 5-thiocarboxamide	19·4
6-Mercapto ribofuranosyl pyrrolopyrimidine 5-carbonitrile	14·2
AMP kinase (14,740)	
6-Hydrazino ribofuranosyl pyrrolopyrimidine	37·0
6-Hydrazino ribofuranosyl purine	37·0
4,5-Dichloro ribofuranosyl pyrrolopyrimidine	16·0
ADP kinase (13,480)	
4-Chloro-6-iodo ribofuranosyl pyrrolopyrimidine	16·0

* Tumor cells were incubated as described in Table 2 with 50 μM hypoxanthine-^{14}C with and without 1 mM test compound. Control apparent activities (cpm) are given in parentheses.

TABLE 6. INHIBITION OF PP-RIBOSE-P ACCUMULATION*

Compounds tested	Concn (μM)	PP-ribose-P (% inhibition)
Nebularine	100	81·9
7-Deazanebularine	100	90·2
4-Dimethylamino-7-β-D-ribofuranosyl pyrrolopyrimidine	1000	82·2
4-Methylamino-7-β-D-ribofuranosyl pyrrolopyrimidine	1000	94·2
4-Amino-7-β-D-(3'-deoxyribofuranosyl) pyrrolopyrimidine	1000	83·3
4-Amino-7-β-D-ribofuranosyl pyrrolopyrimidine 5-carboxamidine	1000	90·1

* Ehrlich ascites tumor cells. 2 per cent by volume, were incubated at 37° with shaking in an atmosphere of air in 2·0 ml calcium-free Krebs–Ringer medium containing 25 mM sodium phosphate buffer, pH 7·4, with 5·5 mM glucose and with and without test compounds. Control PP-ribose-P concentration: 2·3 μmoles/g wet weight of cells.

and hypoxanthine-guanine (EC 2.4.2.8) phosphoribosyltransferases. Table 6 shows the effects of nebularine, 7-deazanebularine and several other ribofuranosyl pyrrolopyrimidines on concentrations of PP-ribose-P in Ehrlich ascites tumor cells incubated with glucose but in the absence of glutamine or naturally occurring purine bases. These compounds, all of which inhibited purine ribonucleotide syntheses, also strongly inhibited PP-ribose-P accumulation under these conditions.

In Table 2, an apparent stimulation of adenylate deaminase activity was calculated because of an increased conversion of radioactive adenine to hypoxanthine and inosine. These metabolites are products of purine nucleotide catabolism, but because effects of drugs on nucleotide synthesis and nucleotide catabolism cannot easily be distinguished under the conditions used, further experiments were conducted to test the effects of nebularine and 7-deazanebularine on adenine nucleotide catabolism. The data of Table 7 show that nebularine caused a slight breakdown of ATP to other adenine ribonucleotides, but did not produce a significant increase in the formation of radioactive inosine plus hypoxanthine. 7-Deazanebularine, in contrast, caused a 2-fold increase in accumulation of radioactive inosine plus hypoxanthine, although this was produced by only a 10 per cent decrease in radioactive ATP concentrations.

Brdar and Reich[11] have recently reported that 7-deazanebularine is converted to mono-, di- and triphosphorylated derivatives in mouse fibroblasts, and preliminary studies of the metabolism of this compound were also conducted using Ehrlich ascites tumor cells. Spectrophotometric studies of incubation media following incubation of cells with 7-deazanebularine showed no evidence for the presence of drug metabolites, although considerable amounts appeared to be taken up by the cells.

One g of Ehrlich ascites tumor cells was then incubated for 60 min as described in Table 1 with 500 μM 7-deazanebularine, and a neutralized perchloric acid extract of cells plus medium was chromatographed on DEAE-Sephadex acetate.[21] Spectrophotometric analysis of the "ATP" peak indicated the presence of 7-deazanebularine metabolites; this peak was then evaporated to dryness *in vacuo* and desalted using Dowex-50-H^+. Thin-layer chromatography on cellulose, using 0·1 M sodium phosphate buffer (pH 6·8)–1 M $(NH_4)_2SO_4$–1-propanol (100:60:2), separated three fluorescent compounds from ATP. These have the spectra of 7-deazanebularine, and presumably represent 7-deazanebularine triphosphate, together with the mono- and

TABLE 7. EFFECT OF NEBULARINE AND 7-DEAZANEBULARINE ON ATP CATABOLISM*

	ATP (μmoles/g cells)	ATP + ADP + AMP (μmoles/g cells)	Hypoxanthine + inosine (μmoles/g cells)
Control	24,150	26,604	2487
Nebularine	22,994	25,828	2533
7-Deazanebularine	21,240	24,203	5290

* Radioactive ATP was synthesized by incubating Ehrlich ascites tumor cells as described in Table 2 with 100 μM adenine-^{14}C. After 30 min, the cells were washed and resuspended in media without adenine-^{14}C, and with and without 100 μM nebularine or 7-deazanebularine. Incubation was continued for 20 min.

diphosphate, which were probably produced during analysis; further characterization was not attempted.

Because only small amounts of 7-deazanebularine were available, chemotherapy experiments using Ehrlich ascites tumor cells *in vivo* were not attempted. However, Fig. 2 shows that this compound was a very potent inhibitor of the growth of cultured human leukemia RPMI 6410 cells. The toxicity of 7-deazanebularine in this system is comparable to that against mouse fibroblasts,[11] although somewhat different dose–response relationships are observed in the two systems. Figure 3 shows that nebularine also inhibited the growth of cultured human leukemia RPMI 6410 cells, although about ten times more nebularine than 7-deazanebularine was required for equivalent growth inhibition.

Preliminary experiments were conducted in attempts to evaluate the significance of inhibition of nucleotide formation (and hence presumably of inhibition of PP-ribose-P accumulation) for the observed inhibition of the growth of RPMI 6410 cells by 7-deazanebularine. Table 8 shows that, under conditions similar to those used to measure growth inhibition, 7-deazanebularine inhibited nucleotide syntheses from

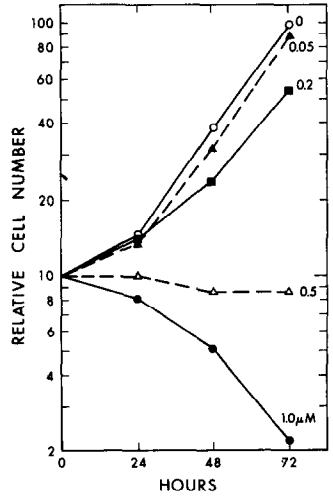


FIG. 2. Growth inhibition by 7-deazanebularine. Human leukemia RPMI cells were grown in RPMI 1620 medium containing 10% fetal calf serum, 100 μg/ml of streptomycin and 100 units/ml of penicillin in the presence of various concentrations of 7-deazanebularine. The initial cell concentration was 100,000/ml.

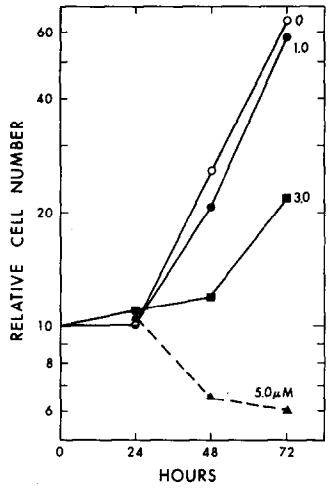


FIG. 3. Growth inhibition by nebularine. Human leukemia RPMI 6410 cells were grown as described in Fig. 2 with various concentrations of nebularine.

hypoxanthine substantially at concentrations of 1·5 and 3·8 μM. These concentrations are only slightly above those at which inhibition of growth was observed.

DISCUSSION

Effects of nebularine and 7-deazanebularine on the metabolism of purine bases in intact tumor cells have not previously been reported, although both compounds have been found to be potent inhibitors of purine biosynthesis *de novo*[16,22] in Ehrlich ascites tumor cells. Both compounds also inhibited partially purified adenosine kinase (EC 2.7.1.20), the enzyme that presumably phosphorylates them;[22–24] 1 mM 7-deazanebularine did not inhibit adenine phosphoribosyltransferase.[25] Previous studies have also investigated the effects of several of the other purine ribonucleosides and ribofuranosyl pyrrolopyrimidines considered here on adenine phosphoribosyltransferase,[22,25] hypoxanthine-guanine phosphoribosyltransferase,[26] adenosine kinase [22,24] and nucleoside uptake into human erythrocytes.[22,27]

TABLE 8. INHIBITION OF NUCLEOTIDE SYNTHESIS BY 7-DEAZANEBULARINE IN RPMI 6410 CELLS.

7-Deazanebularine (μM)	Nucleotide radioactivity (% inhibition)
1·5	31
3·8	74
7·5	91
15	94

* Human leukemia RPMI 6410 cells (6·3 × 10^5 cells/ml) were incubated in RPMI 1620 medium containing 10% fetal calf serum, 100 μg/ml of streptomycin and 100 units/ml of penicillin and various concentrations of 7-deazanebularine. After 20 min, hypoxanthine-^{14}C was added to a final concentration of 20 μM, and incubation continued for 30 min. Control nucleotide radioactivity: 64,220 cpm.

In general, those compounds which inhibited nucleotide synthesis from purine bases in these studies, also inhibited purine biosynthesis *de novo*,[16,22] and inhibition of PP-ribose-P synthesis was suggested as the primary site of action of these ribofuranosyl pyrrolopyrimidines and purine ribonucleosides. Direct measurements of PP-ribose-P concentrations in cells incubated with glucose and these drugs are compatible with this hypothesis. At the present time, the possibility cannot be completely ruled out that the test compounds are converted to purine and pyrrolopyrimidine bases, which then react with P-ribose-P via the purine phosphoribosyltransferases and thereby reduce PP-ribose-P concentrations. However, insofar as it has been tested,[25–29] there is no correlation between inhibitory activity of nucleosides in the present study and affinity for the purine phosphoribosyltransferases. Likewise, purine was not a potent inhibitor of PP-ribose-P accumulation in tumor cells.[15] In fact, there is little information available regarding the conversion of these compounds to bases.

The synthesis of PP-ribose-P from glucose in Ehrlich ascites tumor cells may be inhibited not only by inhibitors of PP-ribose-P synthetase (EC 2.7.6.1), but also by compounds that affect other aspects of glucose metabolism.[20,30,31] Thus, although it seems likely that nebularine and 7-deazanebularine and related compounds inhibit PP-ribose-P synthetase, further studies are required to identify unequivocally their enzymatic site of action.

Previous studies[32] have shown that PP-ribose-P synthesis can be inhibited by a few purine analogs and derivatives that are not converted to nucleotides, but predominantly by compounds that are converted to purine analog nucleoside triphosphates. Both nebularine and 7- deazanebularine apparently are readily converted to triphosphates,[5,6,11] and Brdar and Reich[11] have shown that 7-deazanebularine is incorporated into both RNA and DNA in mouse fibroblasts. Although earlier studies[5] of nebularine metabolism in rat liver *in vivo* did not detect incorporation into nucleic acids, the specific activity of the nebularine used was quite low, and this point deserves to be reinvestigated. Shigeura *et al.*[33] have shown that 6-methylaminopurine ribonucleoside is converted only to the monophosphate derivative in Ehrlich ascites tumor cells, and thus there may be both monophosphate and triphosphate inhibitors of PP-ribose-P synthesis. Whether they act at the same site is not yet known.

Because the cultured human leukemia cells studied were grown in the absence of purine or pyrimidine bases, inhibition of PP-ribose-P synthesis would affect both purine and pyrimidine biosynthesis *de novo*, the processes which under these circumstances provide the purine and pyrimidine nucleotides required for growth. The concentrations of 7-deazanebularine that were required to inhibit nucleotide syntheses (an indirect measure of inhibition of PP-ribose-P accumulation, which cannot be determined directly under these conditions) were two to three times greater than those required to inhibit the growth of cultured human leukemic cells. This suggests that other effects of 7-deazanebularine, such as the observed incorporation of drug in nucleic acids,[11] may be as important or even more important, for growth inhibition than inhibition of PP-ribose-P and nucleotide syntheses. Further experiments are required to evaluate the relative importance of the several biochemical effects of this antimetabolite.

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