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SOME INHIBITORY PROPERTIES OF 6-N-HYDROXYLAMINOPURINE: AN ANALOG OF ADENINE AND HYPOXANTHINE*

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Abstract—6-N-Hydroxylaminopurine (HAP) prolonged the survival time of mice bearing sarcoma 180 ascites cells; several other ascitic neoplasms were less sensitive to this agent. The rate of incorporation of 2-^{14}C-glycine into both polynucleotide adenine and guanine of sarcoma 180 was depressed 90% or more by treatment with HAP, and the utilization of 8-^{14}C-hypoxanthine and 8-^{14}C-adenine for nucleotide formation was decreased by approximately 85% and 50% respectively. HAP competitively inhibited the formation of adenylic acid from adenine in cell-free extracts of sarcoma 180; the inhibition of inosinic acid formation from hypoxanthine by this analog was complex, being only partially competitive.

Dialyzed extracts of either sarcoma 180 or of a variant resistant to purine analogs formed nucleotide from HAP at similar rates, whereas extracts of the L1210 lymphoma formed 5·6-fold more HAP-nucleotide than did those from a 6-mercaptopurine-resistant subline. The extracts from drug-sensitive and -resistant L1210 cells converted adenine to the nucleotide level at equal rates; only the susceptible neoplasm converted guanine to guanylic acid at a significant rate. In cultured L5178Y lymphoblasts, capable of synthesizing purine nucleotides *de novo*, both hypoxanthine and adenine prevented inhibition of cell reproduction by HAP; on a molar basis adenine was the more effective. In amethopterin-treated L5178Y cells, high levels of HAP supported cell growth in the presence of thymidine and serine. The results suggest that HAP may function as both an antagonist of adenine and hypoxanthine in mammalian cells. Furthermore, the inhibition of cell reproduction caused by this compound appears to be attributable to interference with the biosynthesis of purine nucleotides.

THE development of resistance to purine analogs possessing tumor-inhibitory properties is associated, in a considerable number of instances, with the loss or decrease of guanosine-5′-phosphate-inosine-5′-phosphate pyrophosphorylase activity; however, in these neoplastic cells the adenosine-5′-phosphate pyrophosphorylase activity remains unaltered.[1] Consequently, sensitivity to analogs of guanine or hypoxanthine that require conversion to the nucleotide level for inhibitory activity is lost, while sensitivity to purine analogs that are converted to the nucleotide level by adenosine-5′-phosphate pyrophosphorylase is retained.[2–6] The need for potential adenine antimetabolites to overcome resistance to certain purine analogs has been stressed in

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a recent review by Welch;[7] however, compounds with significant antineoplastic activity have not been available for use in the chemotherapy of neoplasms resistant to purine analogs because of altered nucleotide pyrophosphorylase activity. Consequently, we have measured the tumor-inhibitory potency of the potential adenine antagonist, 6-hydroxylaminopurine (HAP),* in purine analog-sensitive and -resistant neoplastic cells and have investigated some of the biochemical properties of this agent in these tumor cells *in vivo* and *in vitro*.

MATERIALS AND METHODS

HAP was synthesized by the method of Giner-Sorolla and Bendich.[8]

Experiments were performed on 9- to 11-week-old female Ha/ICR Swiss mice (A. R. Schmidt Co., Madison, Wis.) or male BDF1 mice (Cumberland View Farms, Cumberland, Tenn.). The following transplanted ascitic neoplasms were employed: sarcoma 180, sarcoma 180/TG, L1210 lymphoma, L1210/MP lymphoma, and L5178Y lymphoma. Transplantation of ascites cells was carried out by withdrawing peritoneal fluid from a donor mouse bearing a 7-day tumor growth. The suspension was centrifuged for 2 min (1,600 *g*), the supernatant peritoneal fluid was decanted, a 10-fold dilution with isotonic saline was made, and 0·1 ml of the cell suspension was injected intraperitoneally into each animal. In all experiments, mice were maintained on Purina laboratory chow and water *ad libitum*. The sensitivity of transplanted neoplasms to HAP was determined by measuring the prolongation of survival time afforded by drug treatments, with techniques previously described.[9] HAP solutions were freshly prepared daily.

Measurement of the degree of inhibition of metabolic pathways involved in the biosynthesis of purine nucleotides was carried out by treating each animal bearing a 6-day ascites cell growth with a single i.p. injection of HAP dissolved in isotonic saline. Fifteen minutes after this dose each mouse was injected i.p. with 100 μg of 2-^{14}C-glycine (Tracer-lab., Waltham, Mass. 3·1 $\times$ 10^4 cpm/μg); 50 μg of 8-^{14}C-adenine (California Corp. for Biochemical Research, Los Angeles, Calif., 2·4 $\times$ 10^4 cpm/μg); or 50 μg of 8-^{14}C-hypoxanthine (Nichem Inc., Bethesda, Md., 3·8 $\times$ 10^3 cpm/μg); and 1 hr was allowed for incorporation. The cells were then harvested, and the nucleic acid purines and acid-soluble adenine were isolated and their specific activities determined.[10, 11]

Nucleotide pyrophosphorylase activity was measured in dialyzed extracts of ascites cells; extracts were prepared by three cycles of alternate freezing and thawing in a dry ice-acetone bath, centrifugation at 100,000 *g* for 1 hr, and dialysis for 18–20 hr against distilled water at 4°. The extract (0·1 ml containing 0·65–0·84 mg protein) was incubated at 37° for 20 min with a purine, 0·75 μmole; phosphoribosylpyrophosphate, 1·5 μmoles; magnesium chloride, 5·0 μmoles; and Tris, 80 μmoles (pH 8·0); total volume, 1·0 ml. Under these conditions substrate levels were optimum and the formation of product was linear for at least 20 min. The reaction was terminated by the addition of 5·0 ml of water at 4° and the mixture was chromatographed on columns (1·2 $\times$ 10 cm) of ECTEOLA-cellulose. Unreacted purine was eluted with water and the nucleotide eluted with 0·2 N HCl. No nucleotide was formed in the absence from the reaction mixture of either enzyme or phosphoribosylpyrophosphate. The reaction product

* The following abbreviations are used: HAP, 6-N-hydroxylaminopurine; GD_{20}, generation dose$_{20}$; NA, nucleic acid; AS, acid-soluble; and S-180, sarcoma 180.

of HAP and phosphoribosylpyrophosphate chromatographed on ECTEOLA-cellulose as a nucleotide and exhibited an ultraviolet spectrum similar to that of HAP with an absorption maximum at 266 mμ; this product was assumed to be the ribonucleotide of HAP. The concentration of nucleotide was determined by radioactivity for adenine and hypoxanthine and spectrophotometrically at 271 mμ for HAP on the assumption that the molar extinction coefficient of the nucleotide does not differ significantly from that of HAP.

Growth studies in culture were carried out on L5178Y lymphoma cells in Fischer medium,* employing methods previously described.[12] Cells were inoculated at a density of 2×10^3 per ml; after 96-hr incubation the cell number was determined with a Coulter particle counter. The unit activity of serum (that amount sufficient to permit half-maximal rate of growth) varied between 0·5 and 1·2%. At the time of initial exposure to HAP and other compounds the cells were in a lag phase, an effect attributable solely to exposure to medium at less than 25°.

TABLE 1. THE EFFECT OF 6-N-HYDROXYLAMINOPURINE ON THE SURVIVAL TIME OF MICE BEARING TRANSPLANTED NEOPLASMS

Neoplasm	Daily dosage* (mg/kg)	Average survival (days)	No. of mice	Average change in body wt.† (%)
Sarcoma 180	0	11·4	15	+15·4
	1	13·0	5	+20·2
	5	17·2	15	+26·6
	10	16·9	15	+23·1
	15	17·4	10	+24·3
	20	15·0	5	+38·6
	30	16·6	10	+24·1
	40	13·0	5	+26·1
Sarcoma 180/TG	0	17·5	10	+29·6
	10	17·1	15	+25·8
	20	15·9	10	+25·9
L1210 Lymphoma	0	6·0	10	
	40	7·0	5	
	60	7·3	10	
	80	8·2	10	
L1210/MP Lymphoma	0	8·1	10	
	40	8·6	5	
	60	8·5	10	
	80	9·0	10	
L5178Y Lymphoma	0	13·3	10	+27·3
	20	12·1	10	+11·9
	40	16·1	10	+6·7
	80	15·6	10	+3·3

* Therapy was initiated 24 hr after implantation of tumor cells and was continued once daily for 6 consecutive days at the indicated daily dosage in all but the L1210 and the L1210/MP lymphomas, with which drugs were administered once daily for 4 consecutive days.

† Average weight change from onset to termination of therapy.

RESULTS

The effects of HAP on the survival time of mice bearing several ascitic neoplasms was used as a measure of the antineoplastic efficacy of this agent; the results are presented in Table 1. HAP caused a consistent lengthening of the life span of mice

* Available at Hyland Laboratories, Los Angeles, Calif.

bearing sarcoma 180 over a relatively wide range of doses (5–30 mg/kg); in results not presented here, adenine (included as a control) did not significantly alter survival in doses of 20–40 mg/kg. No toxicity as measured by weight loss was observed; however, in this neoplasm 40 mg HAP/kg was less effective than were lower doses. Only little or no significant prolongation of the survival time of mice bearing several other ascitic neoplasms was afforded by treatment with HAP.

To ascertain whether the inhibition of neoplastic growth by HAP was associated with metabolic alterations in purine metabolism, the effects of this agent on the incorporation of radioactive glycine, adenine, and hypoxanthine into purine nucleotides of sarcoma 180 ascites cells was measured (Table 2). Treatment with HAP caused a marked (90% or greater) inhibition of the incorporation of glycine into purines,

TABLE 2. THE EFFECT OF 6-N-HYDROXYLAMINOPURINE ON PURINE NUCLEOTIDE BIOSYNTHESIS IN SARCOMA 180 ASCITES CELLS *in vivo*

Isotopic substrate	N-Hydroxylaminopurine	cpm/μmole × 10^{-2}			cpm/mg protein
		NA guanine	NA adenine	AS adenine	
2-^{14}C-Glycine	—	8·3	7·6	154	1,840
	+	0·6	0·4	15	2,300
8-^{14}C-Adenine	—	8·2	89	1,180	
	+	5·5	39	521	
8-^{14}C-Hypoxanthine	—	6·6	7·1	128	
	+	0·9	0·5	9·2	

Tumor-bearing mice were injected with a single dose of hydroxylaminopurine i.p. (50 mg/kg); 15 min later, 100 μg of 2-^{14}C-glycine (3·1 · 10^4 cpm/μg), 50 μg of 8-^{14}C-adenine (2·4 × 10^4 cpm/μg), or 50 μg of 8-^{14}C-hypoxanthine (3·8 · 10^3 cpm/μg) was administered to each mouse, and tumor cells were harvested after being exposed to the radioisotope for a period of 1 hr. Each figure represents the average of results obtained from 4 mice.

while a slight stimulation of glycine incorporation into residual protein was observed. The rate of utilization of 8-^{14}C-adenine for the formation of acid-soluble compounds and of nucleic acids was decreased by about 50%, and the conversion of 8-^{14}C-hypoxanthine to adenine and guanine was markedly depressed (93% and 86% respectively) by exposure of cells to HAP.

Since these data indicate that HAP interferes with the formation of nucleotides from both preformed adenine and hypoxanthine, the effect of HAP on the nucleotide pyrophosphorylase activity of extracts of sarcoma 180 ascites cells was determined; the results were plotted according to the method of Lineweaver and Burk[13] (Figs. 1 and 2). HAP inhibited competitively the formation of adenosine-5′-monophosphate from adenine (Fig. 1); the results depicted in Fig. 2 indicated that the HAP-induced inhibition of inosine-5′-phosphate pyrophosphorylase is of the mixed type.

Table 3 shows the results obtained when the nucleotide-forming capacities of purine analog-sensitive and -resistant neoplastic cells were determined. Cell-free extracts of sarcoma 180 and of a purine analog-resistant variant (sarcoma 180/TG) formed essentially equivalent quantities of HAP-nucleotide. Similar extracts of L1210 and L1210/MP lymphomas formed nucleotide from adenine at equal rates, while the ability to form nucleotide from guanine by extracts of L1210/MP was essentially

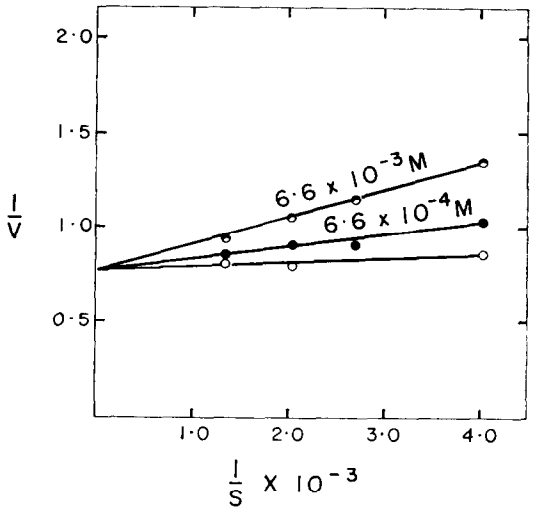

FIG. 1. Kinetic study of the inhibition by hydroxylaminopurine of the adenosine-5′-phosphate pyrophosphorylase activity of sarcoma 180 ascites cells. Varying levels of 8-^{14}C-adenine ($2{\cdot}5 \times 10^4$ cpm/μmole); phosphoribosylpyrophosphate, 1·5 μmoles; magnesium chloride, 5·0 μmoles; Tris, 80 μmoles (pH 8·0); dialyzed extract, 0·1 ml; and hydroxylaminopurine at the indicated concentrations in a total volume of 1·0 ml were incubated at 37° for 10 min. The substrate and the product, adenylic acid, were separated by column chromatography. Velocity was measured as micromoles of radioactive nucleotide formed/mg protein/hr; substrate concentrations are expressed in moles/l.

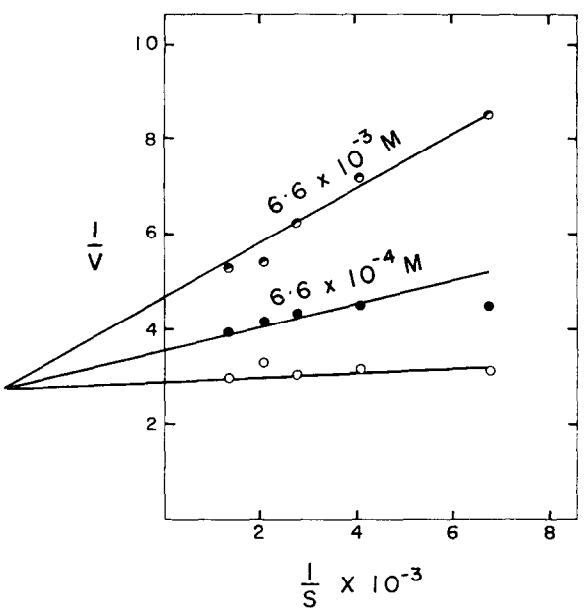

FIG. 2. Kinetic study of the inhibition by hydroxylaminopurine of the inosine-5′-phosphate pyrophosphorylase activity of sarcoma 180 ascites cells. All reactions were carried out as described in Fig. 1 except that 8-^{14}C-hypoxanthine ($5{\cdot}2 \times 10^5$ cpm/μmole), was used in place of the 8-^{14}C-adenine.

lost; these results are in agreement with those of Brockman *et al.*[11] Although both sublines formed the nucleotide of the analog, extracts of L1210 formed 5·6-fold more than did those of L1210/MP.

TABLE 3. THE NUCLEOTIDE-FORMING CAPACITIES OF EXTRACTS OF PURINE-SENSITIVE AND -RESISTANT ASCITES CELLS

Substrate	L1210	L1210/MP	S-180	S-180/TG
	(μmoles nucleotide formed/hr/mg protein)			
Adenine	0·91	0·81		
Guanine	0·95	0·04		
HAP	2·13	0·38	1·38	1·64

The following mixture was incubated at 37° for 20 min at pH 8·0: purine, 0·75 μmole; phosphoribosylpyrophosphate, 1·5 μmoles; magnesium chloride, 5·0 μmoles; Tris, 80 μmoles; and dialyzed extract (0·1 ml containing 0·65 to 0·84 mg protein); total volume 1·0 ml.

HAP caused marked inhibition of the growth of L5178Y lymphoma cells in culture under conditions in which the cells could synthesize purine nucleotides *de novo* (Fig. 3); a concentration of $0{\cdot}2 \times 10^{-5}$ M HAP reduced the number of cell generations undergone to about 80% of that observed with the control cells (GD_{20}). At a level of $1{\cdot}5 \times 10^{-5}$ M hypoxanthine, $1{\cdot}0 \times 10^{-5}$ M HAP was required to produce a GD_{20}. Essentially the same results were obtained with $0{\cdot}15 \times 10^{-5}$ or $0{\cdot}5 \times 10^{-5}$ M hypoxanthine. Adenine ($1{\cdot}5 \times 10^{-5}$ M) was a more effective reversing agent than hypoxanthine, and 20×10^{-5} M HAP was required to produce an equivalent GD_{20}: with

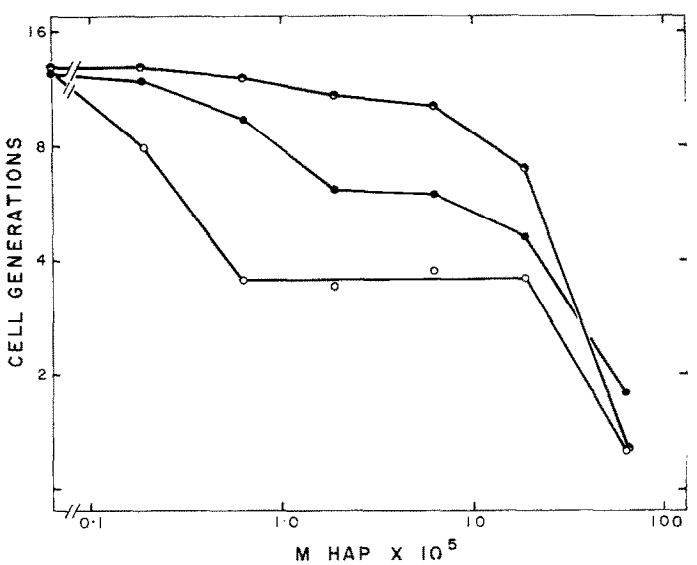

FIG. 3. The effect of hydroxylaminopurine on the growth of cultured L5178Y lymphoblasts capable of synthesizing purine nucleotides *de novo*. Cells were inoculated at a density of 2×10^3/ml into tubes containing the purines and inhibitor. After incubation for 96 hr, the extent of cell growth was determined; ○—○ HAP; ●—● HAP, $1{\cdot}5 \times 10^{-5}$ M hypoxanthine; ◓—◓ HAP, $1{\cdot}5 \times 10^{-5}$ M adenine.

$0{\cdot}15 \times 10^{-5}$ M adenine, less HAP (2×10^{-5} M) was required to produce a GD_{20}. Since HAP behaved as an antagonist of both adenine and hypoxanthine for the reproduction of L5178Y cells, the contribution of the *de-novo* biosynthetic pathway to these phenomena was estimated by establishing conditions under which cells were unable to synthesize purine nucleotides *de novo*. This was accomplished by creating a deficiency of coenyzme forms of tetrahydrofolic acid by the addition of a completely inhibitory level of amethopterin (6×10^{-7} M) to the medium; growth then occurred only in the presence of a utilizable purine, thymidine, and serine.[15] Under these conditions, 5×10^{-5} M hypoxanthine almost completely prevented the toxicity of HAP (Fig. 4); in data not shown, similar results were obtained with $1{\cdot}5 \times 10^{-5}$ M

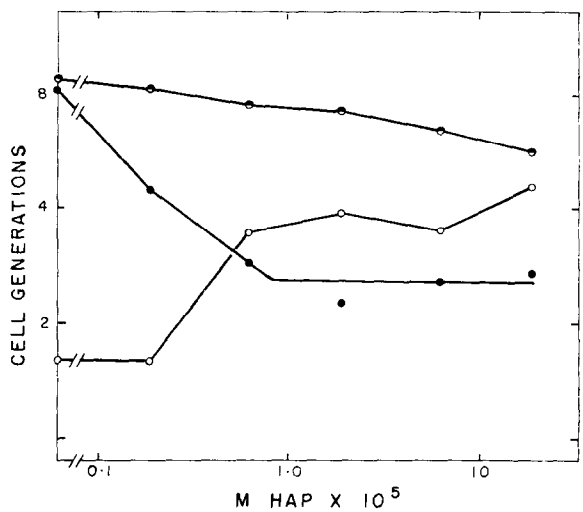

FIG. 4. The effect of hydroxylaminopurine on the growth of cultured L5178Y lymphoblasts in the absence of purine nucleotide biosynthesis *de novo*. Conditions were those described in Fig. 3; ●—● HAP; ○—○ HAP, 6×10^{-7} M amethopterin, 5×10^{-6} M thymidine; ◓—◓ HAP, 6×10^{-7} M amethopterin, 5×10^{-6} M thymidine, 5×10^{-5} M hypoxanthine. In all cases 1×10^{-4} M serine was present in the culture medium.

adenine. In the presence of thymidine and serine, however, HAP supported cell reproduction; this effect was not attributable to interference by HAP ($1\text{–}30 \times 10^{-5}$ M), with the energy-dependent transport of amethopterin[16] (6×10^{-8} M), nor did HAP affect the toxicity of amethopterin for cell reproduction in a medium free from thymidine. Furthermore, in media free from amethopterin, thymidine (1×10^{-7} to 1×10^{-5} M) did not prevent the inhibition of growth produced by HAP.

DISCUSSION

HAP prolonged the survival time of mice bearing sarcoma 180 ascites cells; much less or no inhibition of other transplanted neoplasms was observed in these studies or in those of Giner-Sorolla and Bendich.[8] Earlier work from this laboratory indicated that HAP caused pronounced inhibition of the growth of Ehrlich ascites carcinoma cells *in vivo* and that this inhibitory activity could be partially prevented by the inclusion of 0·2% adenine to the diet.[17] Although these findings suggested that HAP

was partially functioning as an adenine antagonist, the inability of dietary adenine to reverse completely the toxic effects of HAP[17] suggested that other factors were involved. For this reason, the effect of HAP on the utilization of isotopic precursors of purine nucleotides was measured in sarcoma 180 ascites cells. The rate of glycine incorporation into both polynucleotide a denine and guanine was depressed by greater than 90% in cells exposed to HAP. This suggested that HAP can simulate the demonstrated capacity *in vitro* of adenine to function as a feedback inhibitor of purine synthesis *de novo* in ascites-cell systems,[10, 18, 19] presumably acting at the nucleotide level by inhibition of phosphoribosylpyrophosphate amidotransferase.[20] In a similar ascites cell system *in vivo*, adenine at a level of 30 mg/kg did not depress the rate of incorporation of 2-^{14}C-glycine into polynucleotide guanine;[21] however, a sufficiently inhibitory intracellular concentration of adenine (or a nucleotide derivative) at the enzymatic site is probably difficult to maintain *in vivo*.

The conversion of hypoxanthine to adenine and guanine nucleotides in sarcoma 180 ascites cells was markedly inhibited by HAP, and the conversion of adenine to the nucleotide level also was retarded by this agent, although to a lesser extent (Table 2). Measurement of the effects of the analog on the conversion of adenine and hypoxanthine to nucleotides in dialyzed cell-free extracts indicated that HAP competitively inhibits the conversion of adenine to adenylic acid, whereas the inhibition of inosinic acid formation from hypoxanthine was mixed (Figs. 1 and 2). These results suggested that HAP functions as an antimetabolite of both adenine and hypoxanthine, but that its role as an analog of hypoxanthine is complex; possibly it interferes not only with the binding of substrate but with the breakdown of the enzyme–substrate complex.

Extracts of both sarcoma 180 and a purine analog-resistant variant of this neoplasm (sarcoma 180/TG) were able to form HAP nucleotide at similar rates. The development and biochemical properties of sarcoma 180/TG have been described;[22, 23] the resistance of this neoplasm to purine analogs appears to be due to an increased capacity to degrade analog nucleotide and an inability of that analog nucleotide present to inhibit the biosynthesis of guanine nucleotides. In agreement with the findings of Brockman *et al.*,[14] cell-free extracts from the L1210 and L1210/MP lymphomas convert adenine to nucleotide at equal rates, while in the mercaptopurine-resistant subline, guanosine-5′-phosphate pyrophosphorylase activity was essentially absent. The formation of HAP nucleotide by extracts of L1210 lymphoma was 5·6-fold greater than that of the resistant variant, which suggests that either the formation of HAP nucleotide in these extracts is mediated by both the adenosine-5′-phosphate and the inosine-5′-phosphate pyrophosphorylases, or HAP is converted to the nucleotide level by the adenosine-5′-phosphate pyrophosphorylase only, which had a decreased affinity for HAP in extracts of the mercaptopurine-resistant subline. A decision between these alternatives must await future investigation.

In cultured L5178Y lymphoma cells the HAP-induced inhibition of growth was prevented by the addition of either hypoxanthine or adenine to the medium; however, hypoxanthine was less effective than adenine in this respect (Fig. 3). These results may relate to the finding that HAP is a competitive inhibitor of the utilization of adenine by this nucleotide pyrophosphorylase, whereas inhibition of the conversion of hypoxanthine to inosinic acid appears to be partially noncompetitive. The plateau of partial inhibition, which occurs over the broad concentration range of 0·6–10 × 10^{-5} M HAP (Fig. 3), may reflect an instability of the analog. In cells unable to

synthesize purine nucleotides *de novo* because of a deficiency of coenzyme forms of tetrahydrofolic acid resulting from the exposure of the cells to amethopterin, HAP supported growth in the presence of thymidine and serine (Fig. 4). These results suggest that either HAP per se can serve as a utilizable purine for cell reproduction or the analog may be converted, in part, to a utilizable purine. To produce the degree of cell reproduction afforded by HAP under these conditions, a breakdown of this agent to either adenine or hypoxanthine of only about 10% would have been required. In amethopterin-inhibited L5178Y lymphoma cells supplemented with hypoxanthine, thymidine, and serine (Fig. 4), HAP was a less effective inhibitor of cell reproduction than in cells capable of synthesizing purine nucleotides *de-novo*, which were supplemented with an equivalent amount of hypoxanthine (Fig. 3). These results would suggest that the analog-induced blockade of the *de-novo* biosynthetic pathway is associated, in part, with the decreased rate of cell reproduction.

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