METABOLISM OF 6-MERCAPTOPURINE AND THIOINOSINE IN L 1210 CELLS

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6-mercaptopurine and thioinosine were compared in the metabolism in L 1210 cells in order to investigate whether purine nucleoside was directly phosphorylated to purine nucleoside monophosphate. When homogenates from L 1210 cells were in cubated with [8-^{14}C] inosine, the major radioactive product from inosine was hypoxanthine. Inhibition of inosine phosphorylase of homogenates from L 1210 cells by 6-mercaptopurine and thioinosine was observed. When a cell-free extract of L 1210 cells was incubated with [8-^{14}C] inosine or [^{3}H] thioinosine in an ATP regenerating system, the formation of inosinic acid or thioinosinic acid was observed. The reaction showed dependence upon an ATP regenerating system. The experiment in which [8-^{14}C] 6-mercaptopurine and ribose -5-phosphate were added to the reaction mixture and no thioinosinic acid was found ruled out the conversion of thioinosine to thioinosinic acid via 6-mercaptopurine.

These results indicate that inosine kinase might exist in L 1210 cells, but on a level too low to be detected in the presence of ATP regenerating system. Purine nucleoside might be directly phosphorylated to its nucleoside monophoshpate in L 1210 cells.

6-mercaptopurine (6-MP) and thioinosine are documented to be more effective in inhibiting the growth of L 1210 mouse leukemia cells *in vivo* and in treatment of human acute leukemia. The metabolism of 6-MP has been studied extensively in leukemic cells[1~3]. In contrast with 6-MP, little is known about the metabolism of thioinosine in leukemic cells. Thioinosine had a better therapeutic index than 6-MP against the transplanted adenocarcinoma 775 in BDF mouse[4]. This result indicates that the metabolism of thioinosine might differ from that of 6-MP. In order to investigate wether purine nucleoside is directly phosphorylated to its purine nucleoside monophosphate, purine base and purine nucleoside were compared in the metabolism in L 1210 cells.

I. Materials and Methods

Chemicals. All materials used were obtained from Shigma Chemical Co., St. Louis, Mo., U. S. A.. [8-^{14}C] hypoxanthine (20.7 mCi/mmole), [8-^{14}C] inosine (40.0 mCi/mmole) and [^{3}H] thioinosine (2.5 mCi/mmole) were purchased from the Radiochemical Amersham, England.

Isolation of L 1210 cells. L 1210 cells, harvested from mice on the 7th day after i. p. inoculation of 3×10^{6}cells, were washed twice with 0.9% NaCl solution. Contaminating erythrocytes were lysed by adding 3 volumes of water at 0°C. After 40 seconds, isotonicity was restored by addition of 1 volume of 3.6% NaCl solution. The unbroken cells were collected by centrifugation at $500\times g$ for 10 minutes and washed twice with 0.9% NaCl solution by resuspension and centrifugation.

Enzyme preparation. L 1210 cells (2×10^{6}cells) were suspended in 1 ml of ice cold distilled water and homogenized for 40 seconds with a teflon homogenizer. The homogenates were used for the assay of inosine phosphorylase[5], the formation of purine nucleoside from purine base[6] and the assay of ribosyl transfer reaction[5]. L 1210 cells were suspended in 3 volumes of ice cold distilled water and homogenized for 40 seconds with a teflon

homogenizer. After centrifugation at 10,000×g for 10 minutes, the supernatant was separated and used for the direct phosphorylation of purine nucleoside.

Assay of inosine phosphorylase activity. Assay of inosine phosphorylase activity was based on the method described by KIM *et al*[5]. The reaction mixture contained 50 μmoles potassium phosphate buffer. pH 7.4, 0.5 μmoles [8-^{14}C] inosine and homogenates of 1×10^5 to 2×10^5cells in a final volume of 1 ml.

Formation of purine nucleoside from purine base. The reaction mixture contained 50 μmoles Tris buffer, pH 7.4, 0.5 μmole [8-^{14}C] 6-MP, 5 μmole ribose-1-phosphate and homogenates of 2×10^5cells in a final volume of 1 ml[6].

Assay of ribosyl transfer reaction. The above experiments were repeated with 2 μmole inosine in place of ribose-1-phosphate[5].

The direct phosphorylation of purine nucleoside. The reaction mixture was examined by a radiochemical method of PIERRE and LEPAGE[7]. The reaction mixture contained 1 μmoles Tris buffer, pH 7.4, o.1 μmole ATP, 0.5 μmole creatine phosphate, 7.8 μg creatine phosphokinase, 0.5 μmole $MgCl_2$, 0.05 μmole [8-^{14}C] inosine or 0.05 μmole[^{3}H]thioinosine, and 0.1 mg of protein in a final volume of 100 μl.

After 10 minutes incubation at 37℃, the above reaction mixture was stopped by heating the tube in a boiling water for 2 minutes. The denatured protein was sedimented by centrifugation and then aliquots (20 μl) of the supernatant were taken for examination by paperchromatography using the following solvent systems, A : 5% disodium hydrogen phosphate, and B : aqueous propionic acid (44%) - aqueous *n*-butanol (93.8%) (1 : 1). The R_f values of hypoxantine, inosine and inosinic acid (IMP) with solvent system A as an eluent 0.46, 0.66 and 0.90 respectively: using solvent system B as an eluent, the R_f values were 0.70, 0.48 and 0.21, respectively. The R_f values of 6-MP, thioinosine and TIMP with solvent system A as an eluent were 0.43, 0.66 and 0.90, respectively: using solvent system B as an eluent, the R_f values were 0.40, 0.65 and 0.91, respectively. Purine base or purine nucleoside formations showed a linear relationship under the conditions used. Protein was measured by the method of LOWRY *et al*[8].

II. Results

When homogenates from L 1210 cells were incubated with[8-^{14}C]inosine, homogenates can readily metabolize[8-^{14}C]inosine. The major radioactive products from[8-^{14}C]inosine were hypoxanthine. No degradation of the substrates was observed when the homogenates were boiled prior to incubation or when the reaction was terminated at zero time. Inhibition of inosine phosphorylase of homogenates from L 1210 cells by 6-MP and thioinosine was observed. From a plot of the inhibition rate on semilog paper, 50% inhibition was obtained with 1×10^{-3}M 6-MP. When thioinosine was used in place of 6-MP, the result was almost the same. Inosine phosphorylase activity in homogenates of L 1210 cells was much higher than that of other enzymes responsible for purine metabolism reported in previous studies[2] (Table 1).

In order to clarify the metabolism of 6-MP

Table 1 Effects of 6-MP and thioinosine on the conversion of inosine to hypoxanthine by L 1210 cell homogenates

	Concentration (mM)	Hypoxanthine formed (n moles/3×10^7/hr)	Inhibition (%)
None (control)		87.3	
6-MP	0.05	78.5	10
6-MP	0.5	54.2	39
6-MP	1	46.6	47
Thioinosine	0.05	81.2	8
Thioinosine	0.5	76.7	12
Thioinosine	5	26.1	70

The formation of [8-^{14}C] hypoxanthine from [8-^{14}C] inosine in L 1210 cell homogenates was measured. The reaction conditions were the same as those described in "Materials and Methods" except that 6-MP, and thioinosine was added in the concentrations indicated.

Fig. 1 Phosphorylation of [8-^{14}C] inosine by L 1210 cell-free extract. 0.05 μmole [8-^{14}C] inosine were incubated with L 1210 cell-free extract described in "Materials and Methods". Aliquots of the reaction mixture were chromatographied by the solvent system A after denaturation of the protein.

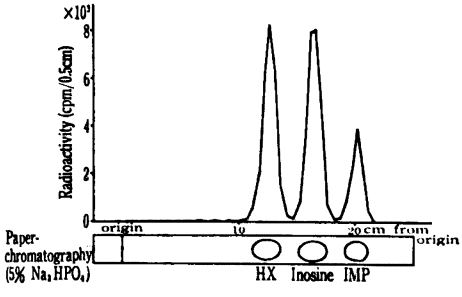
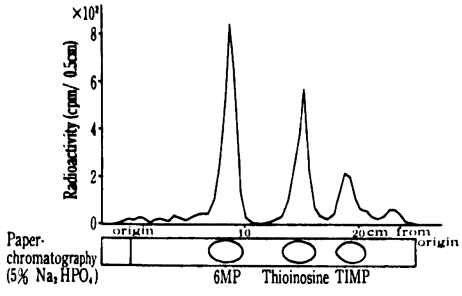


Fig. 2 Phosphorylation of [^{3}H] thioinosine by L 1210 cell-free extract. The reaction conditions were the same as those described in the foot note of Fig. 1 except that [^{3}H] thioinosine was used as the substrate.



itself, [8-^{14}C]6-MP was incubated with ribose-1-phosphate under the same conditions as those described in "Materials and Methods". We could not show conversion of 6-MP to thioinosine. Furthermore, the question of occurence of the ribosyl transfer reaction catalyzed by purine nucleoside phosphorylase is an intriguing one. However, we could not, also, show conversion of 6-MP to thioinosine in the presence of inosine under the same conditions as those described in "Materials and Methods".

A cell-free extract of L 1210 cells was incubated with[8-^{14}C]inosine or[^{3}H]thioinosine in an ATP regenerating system. The reaction mixture was examined by descending paper chromatography, using the solvent system A. Three or four peaks of radioactivity were obtained on the chromatogram (Figs. 1 and 2). These peaks of radioactivity in both chromatograms were identified corresponding to hypoxanthine, inoshine and IMP, or 6-MP thioinosine and TIMP, respectively by exact coincidence of the radioactive peaks with the corresponding carriers. The fourth peak eluted after the peak of TIMP has not been characterized at present. However, it is likely that this peak corresponds to ribose released from[^{3}H]thioinosine. Co-chromatography of the reaction mixture using the solvent system B, also indicated that the products consisted of three or four peaks corresponding to hypoxanthine, inosine and IMP or 6-MP, thioinosine, TIMP and unknown product.

Examination of co-factor requirements for the formation of purine nucleotide indicated that the reaction showed dependence upon an ATP regenerating system. The reaction did not show absolute dependence on ATP as long as the regenerating system was present (Table 2). This was probably due to the presence of endogenous ATP in the extract[7].

Purine nucleotide was cleaved to purine base and ribose-1-phosphate by undergoing phosphorolysis, and then the base was reacted with phosphoribosylpyrophosphate (PRPP) to form nucleotide. That an alternative pathway would not be responsible for the IMP formation was indicated by the results presented in Table 3 and Table 4. The effects of thioinosine on the rate of the phosphorylation of inosine were investigated (Table 5), revealing its inhibitory ones.

III. Discussion

The formation of nucleotide can be mediated either by a nucleoside kinase or by the sequential actions of a nucleoside phosphorylase and a phosphoribosyltransferase. The former pathway is usually condidered not of much significance, since very few nucleoside phosphokinase enzymes have been described in mammalian cells. The findings in the present work are somewhat at variance with certain of above concepts. However, BROKMAN *et al.* indicated that inosine was converted to nucleotide by H. Ep. No. 2 lacking hypoxanthine-guanine phoshporibosyltransferase (HGPRTase)[9]. Furthermore, the presence of an inosine kinase in mammalian cells was first demonstrated by PIERRE *et al*[7]. Recently, PAYNE *et al.* reported the existence of this enzyme in leukocytes of LESCH-

Table 2 Requirements for the phosphorylation of inosine by L 1210 cell-free extract

System	IMP formed (n moles/mg/hr)	Control (%)
Complete	558	100
— cell-free extract	0	0
— ATP	393	70
— creatine phosphate and creatine phosphokinase	36	7
— ATP, creatine phosphate and creatine phosphokinase	0	0

The complete reaction mixture contained 1 μmoles Tris buffer pH 7.4, 0.1 μmole ATP, 0.5 μmole creatine phosphate, 7.8 μg creatine phosphokinase, 0.5 μmole $MgCl_2$, 0.05 μmoles [8-^{14}C] inosine and 0.1 mg protein of cell-free extract. The total volume was 100 μl. The reaction mixture was incubated at 37°C for 10 minutes, and the reaction was stopped by heating the tube as described in "Materials and Methods". After removing denatured protein the supernatant was chromatographied by the solvent system A.

Table 3 Requirements for the phosphorylation of hypoxanthine by L 1210 cell-free extract

System	IMP formed (n moles/mg/hr)
Complete	1560
— cell-free extract	0
— PRPP	0
— PRPP, +ribose-5-phosphate	0

The complete reaction mixture contained 1 μmoles Tris buffer pH 7.4, 0.1 μmole ATP, 0.5 μmole creatine phosphate, 7.8 μg creatine phosphokinase, 0.1 μmole PRPP, 0.5 μmole $MgCl_2$, 0.05 μmoles [8-^{14}C] hypoxanthine and 0.1 mg protein of cell-free extract. The reaction mixture was incubated at 37°C for 10 minutes, and the reaction was stopped by adding 2 μmoles of neutralized EDTA. Aliquots of the reaction mixture were analyzed by a high voltage electrophoresis described in our previous report[2].

Table 4 Requirements for the phosphorylation of 6-MP by L 1210 cell-free extract

System	TIMP formed (n moles/mg/hr)
Complete	602
— cell-free extract	0
— PRPP	0
— PRPP, +ribose-5-phosphate	0

The reaction conditions were the same as those described in Table 3 except that [8-^{14}C] 6-MP was used as the substrate.

Nyhan patients[10].

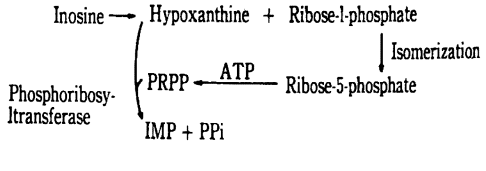
Some of them who have claimed the presence of inosine kinase have not ruled out the alternative pathway shown in Fig. 3. Inosine undergoes to hypoxanthine and ribose-1-phosphate. The latter, after isomerization to ribose-5-phosphate, is converted to PRPP in the presence of ATP. Hypoxanthine reacts with the PRPP thus formed to yield IMP. The experiment in which we added hypoxanthine and ribose-5-phosphate to the reaction mixture and found no IMP would seem to clearly rule out the conversion of inosine to IMP by the alternative pathway shown in Fig. 3. From these studies we conclude that inosine kinase might exist in L 1210 cells, but on a level too low to be detected in the presence of ATP degenerating

Table 5 Effects of thioinosine on the phosphorylation of inosine by L 1210 cell-free extract

Concentration of thioinosine (mM)	IMP formed (n moles/mg/hr)	Inhibition (%)
0	558	0
0.05	342	39
0.5	234	58
5	27	95

The reaction conditions were the same as those described in "Materials and Methods" except that thioinosine was supplemented at the concentrations indicated.

Fig. 3 The conversion of inosine to IMP by the alternative pathway



system.

Clinical trials of thioinosine undertaken by PIERCE *et al.* and WHITTINGTON *et al*[11,12]. showed limited efficacy in the cases resistant to 6-MP. These results may be predictable since it has been shown that thioinosine was rapidly degraded to 6-MP[13]. However, thioinosine has a better therapeutic index than 6-MP against the transplanted Adenocarcinoma 755 in the BDF mouse because of a wider span between effective and toxic doses[4]. These results indicate that the disposition of thioinosin might differ from that of 6-MP. 6-MP must be converted to TIMP to be biologically active. This conversion is catalyzed by HGPRTase[1]. The predominant mechanism of resistance to 6-MP is the loss of HGPRTase. On the other hand, nucleotide synthesis from purine bases is limited by the concentration of PRPP[2]. If a purine nucleoside kinase, which could directly phoshhorylate thioinosine to TIMP, existed in leukemic cells, dependence on the pyrophosphorylase pathway and PRPP might be avoided.

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L 1210 細胞における 6-mercaptopurine と thioinosine の代謝

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purine nucleoside の purine nucleoside monophosphate への直接的燐酸化の可能性を明らかにするため，L 1210 細胞における 6-mercaptopurine と thioinosine との代謝を比較検討した。L 1210 細胞 homogenate と [8-^{14}C] inosine とを incubate したときの代謝産物の大部分は hypoxanthine であった。この反応を触媒する inosine phosphorylase を 6-mercaptopurine や thioinosine は阻害するのが認められた。L 1210 細胞 extract と [8-^{14}C] inosine や [^{3}H] thioinosine を ATP 産生系の存在下で incubate したときには inosinic acid や thioinosinic acid の産生が認められた。この反応系に [8-^{14}C] 6-mercaptopurine と ribose-5-phosphate を添加しても thioinosinic acid の産生は認められなかったことより，thioinosine から 6-mercaptopurine を介しての thioinosinic acid 産生の可能性は除外された。

以上の成績から L1210 細胞には ATP 産生系の関与で測定されうるような極めて低値であるが inosine kinase が存在していることが示唆された。また L 1210 細胞において purine nucleoside monophosphate への直接的な燐酸化の可能性も推定された。