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FORMATION OF 5′-NUCLEOTIDES OF 6-METHYLMERCAPTOPURINE RIBONUCLEOSIDE IN HUMAN TISSUES *IN VITRO*

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Abstract—The recent finding [T. P. Zimmerman, L.-C. Chu, C. J. L. Buggé, D. J. Nelson, G. M. Lyon and G. B. Elion, *Cancer Res.* **34**, 221 (1974)] that 6-methylmercaptopurine ribonucleoside 5′-triphosphate (MMPR-5′-TP) is a human metabolite of both 6-mercaptopurine and azathioprine has prompted a re-examination of the metabolism of 6-methylmercaptopurine ribonucleoside (MMPR) *in vitro*. Human whole blood, peripheral leukocytes and nucleated marrow cells were incubated with MMPR for times as long as 22 hr. Examination of the acid-soluble extracts of these tissues by high-pressure anion-exchange chromatography demonstrated that the 5′-mono-, -di- and -triphosphates of MMPR were formed in all three of these human cell types. As reported previously by others, MMPR (0·2 to 0·9 mM) was taken up rapidly and nearly quantitatively by human blood cells, where it accumulated predominantly as 6-methylmercaptopurine ribonucleoside 5′-monophosphate (MMPR-5′-P). Intracellular concentrations of MMPR-5′-P as high as 2 μmoles/ml of packed erythrocytes were subsequently maintained with little diminution for several hr, during which time MMPR-5′-TP was formed at a linear rate. Relative to MMPR-5′-TP, little of the analog nucleoside 5′-diphosphate accumulated during these incubations. The rate of phosphorylation of MMPR-5′-P was shown to be a function of its intracellular concentration and an apparent K_m of 5·1 mM was estimated with intact erythrocytes. The accumulation of MMPR nucleotides had no discernible effect on the ATP or GTP pools of erythrocytes. The metabolism of MMPR in human leukocytes and marrow cells appeared to be similar in nature to that observed in erythrocytes. In contrast to erythrocytes, however, leukocytes and marrow cells both manifested large (35–85 per cent) decreases in their ATP and GTP pools during incubation with MMPR. Evidence is presented that adenylate kinase (EC 2.7.4.3) is responsible for the phosphorylation of MMPR-5′-P in human erythrocytes.

6-METHYLMERCAPTOPURINE ribonucleoside 5′-phosphate (MMPR-5′-P)* has been identified as a metabolite both of 6-MP[1] and of MMPR.[2] Until recently, further phosphorylation of MMPR-5′-P had not been observed in any tissue,[2–10] and it had become generally accepted that MMPR-5′-P is not metabolized to higher phosphates. However, the recent finding[11] that the 5′-mono-, -di- and -triphosphates of MMPR are human metabolites of both 6-MP and azathioprine has indicated the need for a re-examination of the metabolic reactivity of MMPR-5′-P. Such metabolic

* Abbreviations used: adenylate kinase, ATP:AMP phosphotransferase (EC 2.7.4.3); azathioprine, 6-(1-methyl-4-nitro-5-imidazoyl)thiopurine (IMURAN); guanylate kinase, ATP:GMP phosphotransferase (EC 2.7.4.8); hexokinase, ATP: D-hexose 6-phosphotransferase (EC 2.7.1.1); lactate dehydrogenase, L-lactate: NAD oxidoreductase (EC 1.1.1.27); MMPR, 6-methylmercaptopurine ribonucleoside; MMPR-5′-P, MMPR-5′-DP and MMPR-5′-TP, the 5′-mono-, -di- and -triphosphates of 6-methylmercaptopurine ribonucleoside; 6-MP, 6-mercaptopurine; MPR, 6-thioinosine; nucleoside diphosphokinase, ATP:nucleoside diphosphate phosphotransferase (EC 2.7.4.6); pyruvate kinase, ATP:pyruvate phosphotransferase (EC 2.7.1.40); and venom phosphodiesterase, orthophosphoric diester phosphohydrolase (EC 3.1.4.1).

studies have been conducted with several human tissues and are the subject of the present report.

MATERIALS AND METHODS

Materials. Unlabeled MMPR, phosphoenolpyruvate, penicillin G, streptomycin sulfate and snake venom (*Crotalus adamanteus*) phosphodiesterase were obtained from the Sigma Chemical Co. ATP was from P-L Biochemicals. Hexokinase, lactate dehydrogenase, NADH and pyruvate kinase were purchased from Boehringer Mannheim Corp. CM-Sephadex and Sephadex G-100 were products of Pharmacia Fine Chemicals, Inc. Microgranular DEAE-cellulose (DE-52) was purchased from the Reeve Angel Co. McCoy's 5a medium (modified) with 15% fetal calf serum was from the Grand Island Biological Company and Hanks' balanced salt solution was from Flow Laboratories. MPR was synthesized in this laboratory.[12] [^{14}C]methyl iodide (sp. act., 60·2 mCi/m-mole) was purchased from Amersham/Searle Corp. [Methyl-^{14}C]MMPR was prepared from [^{14}C]methyl iodide and MPR by Dr. Janet L. Rideout of these laboratories according to a published procedure.[3] The [^{14}C]MMPR product of this synthesis was diluted with unlabeled MMPR to a sp. act. of 1·44 mCi/m-mole. MMPR-5′-P was synthesized from MMPR by Dr. Lowrie M. Beacham, III, of these laboratories according to the method of Irie.[13]

Blood. Human blood samples were withdrawn from healthy volunteers into heparinized syringes. Blood samples from laboratory animals were either withdrawn into heparinized syringes or collected into heparinized tubes after decapitation. Blood samples were stored on ice until their use (<2 hr).

Isolation of peripheral leukocytes. Leukocytes were prepared from fresh peripheral blood of a healthy volunteer by a dextran sedimentation method[14] followed by three brief (10-sec) lytic procedures with hypotonic saline to reduce red cell contamination. The final preparation contained approximately 1 per cent red cells. Cells were enumerated by means of a hemacytometer.

Isolation of nucleated marrow cells. A marrow sample from the posterior iliac crest of an unmedicated leukemia (acute granulocytic) patient was suspended in heparinized McCoy's 5a medium. Plasma gel (20%) was added to effect rapid sedimentation of the erythrocytes present. The white cell-rich plasma was subsequently collected and the cells were pelleted by low-speed centrifugation. The cell pellet was suspended in isotonic saline and subjected briefly (20 sec) to two successive lytic procedures with hypotonic saline to eliminate residual contamination by erythrocytes. Cells were enumerated with a hemacytometer.

Incubation conditions and extraction procedure for blood. Fresh heparinized whole blood was supplemented with 44 mM potassium phosphate (pH 7·4), 39 mM D-glucose and 0·5 mg/ml each of penicillin G and streptomycin sulfate, such that the final hematocrit was 39. MMPR was added to the desired concentration. Incubations were carried out at 37° in a Dubnoff metabolic shaking incubator (85 oscillations/min) with air as the gas phase. Under these conditions, no hemolysis was observed during incubation times as long as 22 hr. 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 followed by the addition of 1·00 ml (2×10^4 dis./min) of [^{14}C]AMP. The resulting extracts were clarified by centrifugation and subsequent filtration through Whatman No. 1 paper and were then neutralized with cold, saturated KOH.

After removal of the potassium perchlorate precipitate, the neutralized acid-soluble extracts were evaporated to dryness in a Buchler Evapo-Mix apparatus. The resulting residues were dissolved in 200 μl water and stored at −20° until analyzed in the liquid chromatograph.

Incubation conditions and extraction procedure for leukocytes. Leukocytes were suspended in Hanks' balanced salt solution (supplemented with 10% fetal calf serum, 96 μM hypoxanthine, and 0·6 mg/ml each of penicillin G and streptomycin sulfate) to a concentration of $1{\cdot}1 \times 10^7$ cells/ml, and 5·0-ml portions of this cell suspension were delivered to each of six tissue culture plates (60 mm dia, from Falcon Plastics, Los Angeles, Calif.). Appropriate volumes of 40 mM MMPR were added to give duplicate sets of plates with concentrations of 0, 40 or 380 μM MMPR in the medium. The resulting cell suspensions were incubated at 37° under a 5% carbon dioxide/95% air atmosphere. Incubations were terminated after 4 or 19 hr by chilling the plates on ice. Cells were removed from the plates with a rubber policeman and washed two times with cold isotonic saline. After resuspension in cold saline (0·1 ml), the cells were mixed with 10 ml cold 0·5 M perchloric acid followed by the addition of 0·50 ml (1×10^4 dis./min) [^{14}C]AMP. The resulting extracts were then clarified, neutralized and concentrated (to a final volume of 50 μl) as described above for the blood extracts.

Incubation conditions and extraction procedures for marrow cells. Marrow cells were suspended in McCoy's 5a medium (supplemented with 95 μM hypoxanthine, and 0·5 mg/ml each of penicillin G and streptomycin sulfate) to a concentration of $6{\cdot}2 \times 10^7$ cells/ml, and a 5·0-ml portion of this cell suspension was delivered to each of seven tissue culture plates. MMPR was added to give duplicate sets of plates with concentrations of 0, 76 or 930 μM MMPR in the medium. One plate of cells (no MMPR added) served as a zero-time sample and the cells therefore were immediately washed and extracted with perchloric acid. The remaining six plates of cells were incubated at 37° under a 5% carbon dioxide/95% air atmosphere. Incubations were terminated after 3·5 or 20 hr by chilling the plates on ice. Cells were harvested, washed and extracted as described above for the leukocyte procedure. In this case, the final acid-soluble extracts were concentrated to 200 μl.

Analysis of tissue extracts in high-pressure liquid chromatograph. Acid-soluble tissue extracts were analyzed in a Varian Aerograph model LCS-1000 high-pressure liquid chromatograph equipped with a 1 mm × 3 m Reeve Angel column containing the pellicular anion-exchange resin AS-PELLIONEX-SAX. An auxiliary u.v. (280 nm) flow monitor (Laboratory Data Control model 1280) was connected in series with the standard 254 nm monitor to allow simultaneous recording at the two wavelengths with a Honeywell Electronik 194 two-pen recorder. A 10-μl sample of each tissue extract was injected into the liquid chromatograph. Full-scale absorbance ranges of 0·08 or 0·16 O.D. unit were employed. The eluents were 0·67 mM potassium phosphate–3·3 mM potassium acetate (pH 5·0) and 1·0 M potassium phosphate (pH 3·9). The starting volume of low-concentration eluent in the mixing chamber was 40 ml. The column flow rate was 24 ml/hr and the gradient flow rate 12 ml/hr. A 15-min gradient delay was employed. The oven temperature was maintained at 70°.

Peaks were identified by comparison with the retention times of authentic nucleotide standards and by their 254/280 absorbance ratios. Nucleotide concentrations in the tissue extracts were calculated using response factors (u.v. peak area, in^2/μmole

of nucleotide) determined by injecting known amounts of authentic nucleotide standards into the liquid chromatograph. An authentic MMPR-5′-P standard was used to determine the response factor for the MMPR nucleotides. The content of [^{14}C]AMP in each tissue extract served to normalize each analysis to the original tissue volume (cell count).

Preparation of [methyl-^{14}C]MMPR-5′-P. Fresh human blood (177 ml) was incubated at 37° for 22 hr with 1·3 mM [methyl-^{14}C]MMPR (sp. act., 1·44 mCi/m-mole), inorganic phosphate, glucose and antibiotics as described above. The incubation was terminated by the addition of 5 vol. of cold 0·5 M perchloric acid. The extract was clarified by centrifugation and neutralized with fresh KOH. After removal of the potassium perchlorate precipitate, the neutralized extract was concentrated to 200 ml in a rotary evaporator and chromatographed on an enlarged version of the DEAE-cellulose column (1·8 × 80 cm, total gradient volume = 4000 ml) described by Rustum and Schwartz.[15] The peak of [^{14}C]MMPR-5′-P was eluted shortly after AMP. After adsorption to and elution from charcoal,[16] the concentrated sample of [^{14}C]MMPR-5′-P was further purified by sequential chromatography on Whatman No. 3MM paper (R_f = 0·82) and on a cellulose thin-layer plate (0·5 mm, from Quantum Industries, Fairfield, N.J.) (R_f = 0·85) with *t*-amyl alcohol–formic acid–water (3:2:1), followed by chromatography on Whatman No. 3MM paper (R_f = 0·18) with *i*-propyl alcohol–concentrated ammonium hydroxide–water (7:1:2). The final preparation of [^{14}C]MMPR-5′-P (25 μmoles) was >99 per cent radiochemically pure and was eluted as a single u.v. peak with the same retention time as authentic MMPR-5′-P when analyzed in the high-pressure liquid chromatograph.

Purification and assay of human erythrocytic adenylate and guanylate kinases. Enzymes were purified from outdated human blood obtained from a local hospital. Adenylate kinase was prepared by a modified procedure of Brownson and Spencer[17] in which the second CM-Sephadex column was eluted with a linear gradient of NaCl (100–250 mM) instead of elution with 150 mM NaCl, as originally described. The resulting preparation of adenylate kinase had a sp. act. of 1·3 units/mg of protein and contained no detectable guanylate kinase activity.

Guanylate kinase was prepared by a modified procedure of Agarwal *et al.*[18] After chromatography on DEAE-cellulose, the enzyme was precipitated with ammonium sulfate (added to 80 per cent saturation), dissolved in 5 mM potassium phosphate (pH 7·5), applied to a Sephadex G-100 column (2·5 × 35 cm) and eluted with the same buffer. The fractions with maximal activity were pooled, mixed with ammonium sulfate to saturation and stored at −20° until use. This preparation of guanylate kinase had a sp. act. of 1·9 units/mg of protein and was completely free of detectable adenylate kinase activity.

The assays for adenylate kinase and guanylate kinase, as well as the protein determinations, were carried out according to the procedures described by Agarwal *et al.*[18] An enzyme unit is defined as that amount of enzyme which converts 1 μmole substrate/min at 30°.

Measurement of activity of [^{14}C]MMPR-5′-P as substrate for adenylate and guanylate kinases. The reaction conditions were the same as those employed above for the spectrophotometric enzyme assays with the following exceptions: (1) 6·2 mM [^{14}C]MMPR-5′-P (sp. act., 1·44 mCi/m-mole) replaced the 0·1 mM AMP or GMP; (2) total reaction volumes were 80 μl; and (3) reactions were carried out at 22° instead

of at 30°. The reactions contained either 0·16 unit of adenylate kinase or 0·13 unit of guanylate kinase, as measured by the spectrophotometric assays at 30°. Reactions were allowed to proceed for 19 hr. During this time, samples (3 μl from the adenylate kinase reaction, 5 μl from the guanylate kinase reaction) were withdrawn at intervals, applied to a cellulose thin-layer plate and developed in *t*-amyl alcohol–formic acid–water (3:2:1). In this solvent system, the R_f values for MMPR, MMPR-5′-P, MMPR-5′-DP and MMPR-5′-TP were 0·85, 0·66, 0·37 and 0·27 respectively. The areas corresponding to the 5′-di- and -triphosphates of MMPR were cut out and counted in a liquid scintillation counter. The 19-hr values for [^{14}C]MMPR-5′-DP and [^{14}C]MMPR-5′-TP formation determined by thin-layer chromatography were subsequently verified by high-pressure liquid chromatography. In the reaction involving adenylate kinase, less than 0·2 per cent of the [^{14}C]MMPR-5′-P was cleaved to the nucleoside in 19 hr, while in the parallel incubation with guanylate kinase 4 per cent of the substrate was converted to its nucleoside.

RESULTS

Time dependence of formation of MMPR nucleotides in blood. In initial experiments, fresh blood samples from several healthy individuals were incubated overnight with MMPR as described under Methods. Analysis of the acid-soluble nucleo-

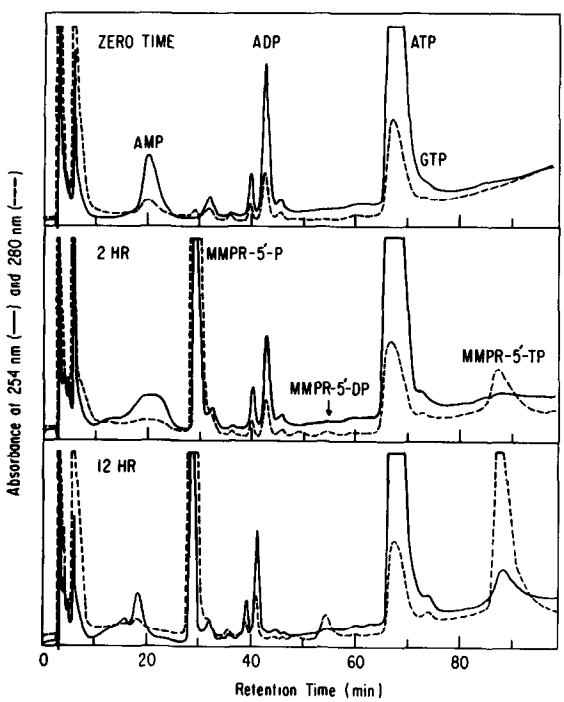

FIG. 1. Progressive synthesis of MMPR nucleotides in human whole blood. A time-course was carried out by incubating fresh human blood (16 ml) with MMPR (869 μM), inorganic phosphate, glucose and antibiotics as described in Materials and Methods. Samples (2·0 ml) of this incubation mixture were withdrawn at 0, 2, 4, 8, 12 and 22 hr after the start of the incubation, and acid-soluble extracts of these samples were prepared and analyzed in the high-pressure liquid chromatograph. Chromatograms of the acid-soluble extracts of the 0-, 2- and 12-hr samples from this kinetic study are presented. The full-scale absorbance was 0·16 O.D. unit for both photodetectors (254 and 280 nm).

tides from these blood samples in the high-pressure liquid chromatograph gave evidence for the formation of the 5′-mono-, -di- and -triphosphates of MMPR in all of these samples. Subsequently, a kinetic study of the formation of these analog nucleotides in human blood was carried out over a 22-hr incubation period. Selected chromatograms of blood extracts prepared at three different times during this kinetic study are presented in Fig. 1. The progressive appearance of three new peaks, exhibiting high 280/254 nm absorbancy ratios characteristic of MMPR nucleotides,[11] is evident in the nucleotide region of the chromatograms. These three metabolites of MMPR *in vitro* were eluted from the liquid chromatograph with the same retention times as the three metabolites of 6-MP *in vivo* previously identified in leukemic blood as MMPR-5′-P, MMPR-5′-DP and MMPR-5′-TP.[11] The metabolite peaks *in vitro* were collected as they were eluted from the liquid chromatograph and were found to exhibit the same u.v. spectral characteristics as MMPR. The metabolite of MMPR *in vitro* eluting with the retention time of MMPR-5′-TP was shifted quantitatively to the retention time of MMPR-5′-DP after treatment of a blood extract with yeast hexokinase plus glucose,[19] and to the retention time of MMPR-5′-P after treatment with snake venom phosphodiesterase. In a similarly consistent manner, the metabolite of MMPR *in vitro* eluting with the same retention time as MMPR-5′-DP was shifted quantitatively to the retention time of MMPR-5′-TP after treatment of a blood extract with pyruvate kinase plus phosphoenolpyruvate, and was also sensitive to treatment with venom phosphodiesterase. Furthermore, peaks of radioactivity were eluted coincidentally with these three metabolite peaks when blood was incubated with [^{14}C]MMPR. Collectively, these criteria were considered sufficient to identify these metabolites of MMPR *in vitro* as the 5′-mono-, -di- and -triphosphate derivatives of this analog purine ribonucleoside.

The full time-course for the formation of MMPR-5′-P, MMPR-5′-DP and MMPR-5′-TP *in vitro* from MMPR in human blood is presented in Fig. 2. Greater than 90 per cent of the MMPR (0·87 mM) added to the incubation was taken up and

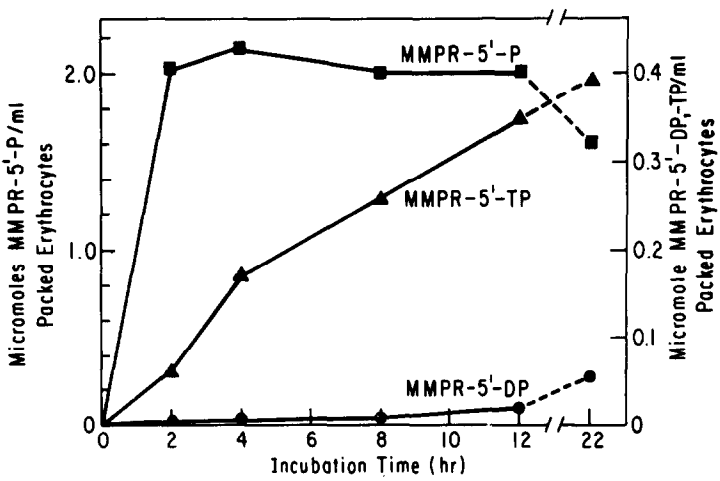

FIG. 2. Time-course of formation of MMPR nucleotides in human whole blood during incubation with MMPR. These data were derived from the experiment described in the legend for Fig. 1. Intracellular concentrations of MMPR nucleotides were calculated from the peak areas of the high-pressure liquid chromatograms as described in Materials and Methods.

phosphorylated (mostly to the 5′-monophosphate) within 2 hr. During the subsequent 10 hr of incubation, the intracellular concentration of MMPR-5′-P changed little from its 2-hr value (2·04 μmoles/ml of packed erythrocytes). During the first 12 hr of incubation, the formation of MMPR-5′-TP proceeded in nearly linear manner. Between 12 and 22 hr, an additional small increase in intracellular MMPR-5′-TP (to 390 nmoles/ml of packed erythrocytes) was observed. Relatively little MMPR-5′-DP accumulated in the blood cells.

It was of interest to investigate the effect of the MMPR nucleotides on the purine ribonucleotide metabolism of human blood cells. For this purpose a second blood incubation, identical in all ways except for the absence of MMPR, was carried out simultaneously in parallel with the kinetic study described in Fig. 2. During the first 8 hr of incubation, the ATP concentration in the MMPR-treated cells was depressed 18–32 per cent relative to that of cells incubated in the absence of MMPR; by 12 hr this difference in ATP levels had vanished. No difference in GTP levels was observed at any time during the parallel 22-hr incubations (±MMPR).

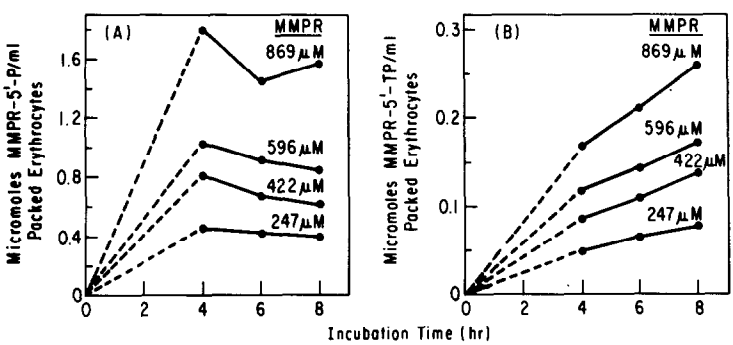

FIG. 3. Time-course of intracellular concentrations of (A) MMPR-5′-P and (B) MMPR-5′-TP in human whole blood during incubation with different concentrations of MMPR. The input concentrations of MMPR in the blood incubation mixtures were 247, 422, 596 and 869 μM. A time–course was carried out at each of these four concentrations of MMPR by incubating samples (10 ml) of fresh human blood with the specified amount of MMPR, inorganic phosphate, glucose and antibiotics, as described in Materials and Methods. Samples (2·0 ml) of these incubation mixtures were withdrawn at 4, 6 and 8 hr after the start of the incubations, and acid-soluble extracts of these samples were prepared and analyzed in the high-pressure liquid chromatograph. Intracellular concentrations of MMPR-5′-P and MMPR-5′-TP were calculated from the peak areas of the chromatograms.

Although not strictly related to the subject of this report, it should be mentioned that whole blood from rats, mice, rabbits and dogs also formed MMPR-5′-P, MMPR-5′-DP and MMPR-5′-TP during overnight incubation with MMPR.

Effect of intracellular concentration of MMPR-5′-P on its rate of phosphorylation in blood cells. Abbreviated time-course plots for the erythrocytic concentrations of MMPR-5′-P and MMPR-5′-TP were determined for blood incubation mixtures supplemented with four different input levels of MMPR (247–869 μM). The results from this experiment, presented in Fig. 3, revealed four characteristics of erythrocytic metabolism of MMPR-5′-P which are especially noteworthy: (1) the intracellular concentrations of MMPR-5′-P varied in relation to the different input concentrations of MMPR (Fig. 3A); (2) the intracellular levels of MMPR-5′-P declined less than 25 per cent during the 4-hr experimental period under consideration (Fig.

3A); (3) the rates of formation of MMPR-5′-TP were linear in all cases during the 4-hr experimental period (Fig. 3B); and (4) the rates of phosphorylation* of MMPR-5′-P were dependent upon the intracellular concentrations of this analog 5′-monophosphate. The data presented in Fig. 3 were used to calculate both the average intracellular concentration of MMPR-5′-P (expressed in μmoles/ml of packed erythrocytes) and the rate of intracellular formation of MMPR-5′-TP (expressed in μmoles/hr/ml of packed erythrocytes) for each of the four different blood incubation mixtures. These latter values were then subjected to analysis by the computer program of Cleland[20] for enzymatic kinetic data and were replotted in double-reciprocal form in Fig. 4. This computational procedure yielded an apparent intracellular K_m of 5·1 mM for MMPR-5′-P and an apparent intracellular V_{max} of 98 nmoles MMPR-5′-TP formed/hr/ml of packed erythrocytes.

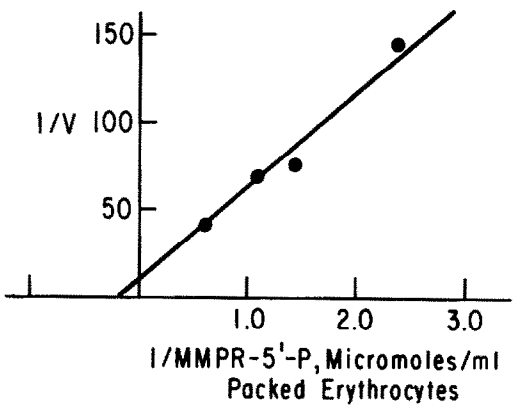

FIG. 4. Plot of reciprocal of rate of formation of MMPR-5′-TP (expressed as μmoles/hr/ml of packed erythrocytes) vs the reciprocal of the average intracellular concentration of MMPR-5′-P (expressed as μmoles/ml of packed erythrocytes). These experimental values were calculated from the data presented in Fig. 3.

Formation of MMPR nucleotides in peripheral leukocytes. The presence of MMPR-5′-TP was demonstrable in the acid-soluble extract of leukocytes which had been incubated for 19 hr in medium containing 380 μM MMPR (Table 1). At the lower concentration (40 μM) of MMPR or at the shorter incubation time (4 hr), no MMPR-5′-TP was detectable. As observed with human erythrocytes, there was an early accumulation of MMPR-5′-P in the leukocytes followed by the gradual formation of MMPR-5′-TP. Relatively little MMPR-5′-DP accumulated in these cells. In contrast to the case with erythrocytes, incubation of leukocytes with MMPR resulted in considerable (40–60 per cent) depression of the intracellular pools of ATP and GTP (Table 1).†

Formation of MMPR nucleotides in nucleated marrow cells. Human marrow cells contained readily detectable levels of MMPR-5′-TP within 3·5 hr of incubation with

* Since little MMPR-5′-DP accumulated in these incubations, the rate of phosphorylation of MMPR-5′-P was reflected directly in the rate of formation of MMPR-5′-TP (cf. Fig. 2).

† These data on endogenous nucleotide pools (Tables 1 and 2) were derived from single experiments; however, in the experience of this laboratory, the expected variation in nucleotide pool sizes determined by this methodology is less than 10 per cent for replicate samples.

TABLE 1. FORMATION OF MMPR NUCLEOTIDES FROM MMPR IN HUMAN PERIPHERAL LEUKOCYTES *in vitro**

Compound	4-Hr incubation samples (nmoles/10^6 cells) MMPR concn in medium (μM)			19-Hr incubation samples (nmoles/10^6 cells) MMPR concn in medium (μM)		
	0	40	380	0	40	380
AMP	0·41	0·22	0·32	0·21	0·23	0·14
ADP	0·18	0·12	0·18	0·08	0·04	0·04
ATP	0·63	0·41	0·36	0·76	0·46	0·31
GTP	0·09	0·14	0·05	0·28	0·17	0·14
MMPR-5′-P		0·18	0·26		0·20	0·36
MMPR-5′-DP		ND†	ND		ND	0·02
MMPR-5′-TP		ND	ND		ND	0·07

* Experimental conditions are described in Materials and Methods.

† ND means not detectable; lower limit of detectability in this experiment was approximately 0·01 nmole MMPR nucleotide/10^6 cells.

TABLE 2. FORMATION OF MMPR NUCLEOTIDES FROM MMPR IN HUMAN NUCLEATED MARROW CELLS *in vitro**

Compound	Zero time sample	3·5-Hr incubation samples (nmoles/10^6 cells) MMPR concn in medium (μM)			20-Hr incubation samples (nmoles/10^6 cells) MMPR concn in medium (μM)		
		0	76	930	0	76	930
AMP	0·09	0·02	0·07	0·08	0·03	0·09	0·04
ADP	0·15	0·09	0·12	0·05	0·10	0·06	0·02
ATP	0·71	0·83	0·66	0·46	1·23	0·23	0·18
GTP	0·18	0·13	0·17	0·14	0·20	0·12	0·13
MMPR-5′-P			0·62	0·72		0·17	1·09
MMPR-5′-DP			0·10	0·02		0·01	0·02
MMPR-5′-TP			0·05	0·05		0·02	0·08

* Experimental conditions are described in Materials and Methods.

MMPR (Table 2). As with the other cell types examined in this study, the 5′-monophosphate was the most abundant nucleotide of MMPR found in these marrow cells. Similar to the finding with leukocytes, incubation of marrow cells with MMPR resulted in marked (35–85 per cent) depression of ATP and GTP pools.

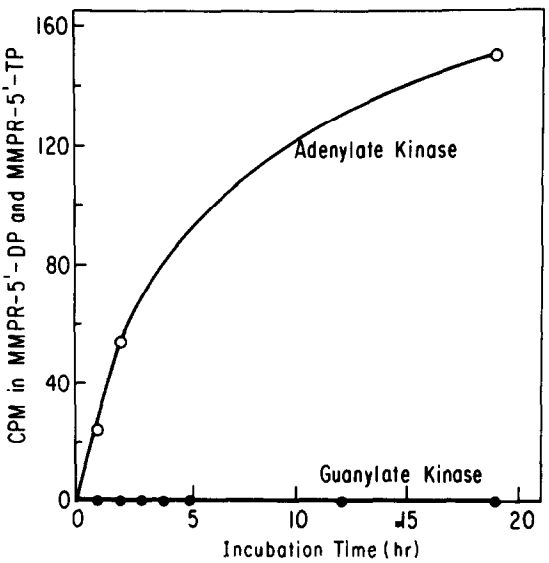

FIG. 5. Reactivity of [^{14}C]MMPR-5′-P with partially purified adenylate and guanylate kinases from human erythrocytes. Each of the two 80-μl reaction mixtured contained: 6·2 mM [^{14}C]MMPR-5′-P (sp. act., 1·44 mCi/m-mole); 2·0 mM ATP; 3·0 mM phosphoenolpyruvate; 0·3 mM NADH; 25 mM $MgCl_2$; 10 mM KCl; 150 mM Tris-acetate (pH 7·5); 10 μg each of pyruvate kinase and lactate dehydrogenase; and either 0·16 unit of adenylate kinase or 0·13 unit of guanylate kinase. The reactions were conducted at 22°. Samples (3 μl from the adenylate kinase reaction, 5 μl from the guanylate kinase reaction) were withdrawn at the indicated times and analyzed for the formation of [^{14}C]MMPR-5′-DP and [^{14}C]MMPR-5′-TP as described in Materials and Methods.

Reactivity of [^{14}C]MMPR-5′-P with human erythrocytic adenylate and guanylate kinases. Partially purified adenylate kinase from human erythrocytes exhibited a low but measurable activity toward [^{14}C]MMPR-5′-P (Fig. 5). The initial velocity of phosphorylation of [^{14}C]MMPR-5′-P (6·2 mM) by adenylate kinase was only $6{\cdot}7 \times 10^{-5}$ times that of AMP (0·1 mM). Under otherwise identical conditions, a comparable amount (i.e. units of enzyme) of partially purified guanylate kinase did not catalyze the formation of detectable quantities of [^{14}C]MMPR-5′-DP or [^{14}C]MMPR-5′-TP. During the 19-hr assay period, adenylate kinase lost 50 per cent of its original activity, while guanylate kinase retained full activity. MMPR-5′-P was found to be a competitive inhibitor with respect both to AMP (with adenylate kinase) and to GMP (with guanylate kinase), with inhibition constants of 14 and 5·7 mM respectively. K_m values of 0·23 and 0·025 mM were determined for AMP and GMP, respectively, with their related kinases. Poor substrate reactivity and paucity of radioactively labeled MMPR-5′-P precluded further enzymatic studies.

DISCUSSION

It has been possible to demonstrate the formation of MMPR-5′-TP *in vitro* in all three of the human cell types examined in this study. The success of the present study can be attributed both to the lengthy incubation times employed and to the sensitivity of the analytical method available with the high-pressure liquid chromatograph, particularly with the 280 nm u.v. flow monitor. The inability of previous studies to detect the formation of the higher phosphates of MMPR was probably due to a deficiency in one or both of these two critical factors.

The intracellular phosphorylation of MMPR-5′-P appears to be mediated by adenylate kinase. Studies carried out both with intact erythrocytes and with partially purified erythrocytic adenylate kinase indicate that this enzyme has a low affinity for MMPR-5′-P and that phosphorylation of MMPR-5′-P occurs at an extremely low rate ($< 10^{-4}$ the rate of phosphorylation of AMP). These two factors dictate the need for maintaining high levels of intracellular MMPR-5′-P in order for phosphorylation of MMPR-5′-P to take place to any appreciable extent. The immediate product of this reaction, MMPR-5′-DP, was not observed to accumulate in these tissues. High levels of nucleoside diphosphokinase are present in human erythrocytes[18] and in peripheral lymphocytes;[21] either this enzyme or pyruvate kinase may be responsible for the rapid subsequent conversion of MMPR-5′-DP to MMPR-5′-TP.

The apparent differential effect of MMPR on the purine nucleoside triphosphate pools of these three tissues is difficult to interpret at present. Consideration of the K_i value (14 mM) for MMPR-5′-P and the K_m value for AMP (0·23 mM), and the fact that the intracellular concentration of AMP is approximately at its K_m value (Tables 1 and 2), renders it unlikely that the observed intracellular levels of MMPR-5′-P are sufficient to inhibit significantly adenylate kinase in these nucleated cells. This difference among cell types would not seem to be related to "pseudo-feedback" inhibition of purine *de novo* biosynthesis by MMPR-5′-P;[22] erythrocytes lack this metabolic pathway,[23,24] and hypoxanthine (96 μM) was added to the incubation media for the leukocytes and marrow cells as an alternate source of purine in order to circumvent this particular metabolic blockade.[25] It is possible that this apparent difference between erythrocytes and nucleated cells may simply reflect differences in experimental conditions (e.g. incubation in whole plasma vs incubation in a synthetic medium, damage incurred by the nucleated cells during their isolation, or differences in total input MMPR per total cell volume in the various incubations). However, the fact that MMPR has been found to cause a reduction in purine nucleotide pools in other tissues[26,27] suggests that the present finding is not of an artifactual nature. Perhaps a more plausible explanation is that leukocytes and marrow cells depend heavily upon oxidative phosphorylation for their energy production[28] and that MMPR or one of its nucleotides interferes with this metabolic process.

The lengthy (20-hr) incubation times employed in this study appear to be essential to a model system *in vitro* which attempts to reproduce the metabolism of MMPR *in vivo*. The half-time of MMPR-5′-P in human blood has been reported to be 4–6 days.[6] In the case of 6-MP and azathioprine, continued daily dosage with these metabolic precursors of MMPR-5′-P would further act to ensure the maintenance of tissue levels of MMPR-5′-P. On the basis of the present results, these sustained levels of MMPR-5′-P *in vivo* would be expected to lead to the slow but continuous forma-

tion of MMPR-5′-DP and MMPR-5′-TP, a clinical finding which has been described recently.[11]

At this time, one can only speculate as to whether these oligophosphate metabolites of MMPR play a role in the inhibitory effects of MMPR on cell proliferation. Possible loci of action for MMPR-5′-DP or MMPR-5′-TP include inhibition of ribonucleotide reductase and of the nucleic acid polymerases, as well as incorporation into nucleic acids.

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