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CELLULAR UPTAKE OF PURINE ANTIMETABOLITES *IN VITRO*—I

UPTAKE OF 6-METHYLTHIOPURINE RIBONUCLEOSIDE BY HUMAN ERYTHROCYTES*

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Abstract—6-Methylthiopurine ribonucleoside (MMPR) is preferentially taken up *in vivo* by human erythrocytes. An uphill transport into the cells against a radioactivity gradient is observed when human whole blood is incubated with radioactive MMPR-^{35}S. The transport obeys Michaelis–Menten kinetics in a drug concentration range of 0·05 to 0·3 mM in whole blood. The average K_m is 0·91 mM at 37°. The transport mechanism is saturable. Inhibition has been demonstrated with MMPR analogs but not with nonspecific inhibitors, thus suggesting that no metabolic energy is consumed. Since intracellularly all of the radioactivity resides in the phorphorylated product, MMPR 5′-monophosphate, rather than in the unchanged MMPR, the transport is uphill with respect to radioactivity but downhill with respect to MMPR. The accumulation and persistence of radioactivity in the erythrocytes are therefore explainable by the intracellular biotransformation of MMPR to the more ionizable and hence less diffusible ribonucleotide.

6-METHYLTHIOPURINE ribonucleoside (6-methylthio-9-β-D-ribofuranosylpurine; NSC-40774, MMPR), an analog of adenosine, is active against experimental tumors resistant to 6-mercaptopurine (NSC-755, MP).[1] Also, MMPR and MP are synergistic in several transplantable rodent tumors.[2, 3] MMPR is ineffective in human leukemia refractory to MP; in combination with MP, it has produced a greater rate of remission in adult acute myelogenous leukemia than has hitherto been achieved with MP alone.[1, 4]

The clinical pharmacology of MMPR and MP has been studied by colorimetric and radiochemical techniques.[5] The biological half-time of radioactivity in human plasma is 5 days for MMPR, but 3 hr for MP. Moreover, in the case of MMPR, the excretion of radioactivity is protracted at an almost constant rate of 10 per cent of the injected dose per day for the first 5 days. In contrast, in the case of MP, the excretion radioactivity is rapid initially, about 65 per cent of the injected dose in 6 hr. Evidently MMPR is temporarily stored in some body compartment from which it exists slowly. An examination of the erythrocytes (RBC) after MMPR-^{35} S administration reveals that the distribution ratio of radioactivity *in vivo* in packed RBC (corrected for

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extracellular water[6]) to that in plasma is 40 to 1. Such a phenomenon is not observed in similar studies with MP-^{35} S; at plasma peak of radioactivity, the distribution ratio of radioactivity in packed RBC to that in plasma is 1 to 100, a 4000-fold difference from MMPR-^{35} S.

In view of the above, we have studied the uptake of MMPR-^{35} S *in vitro* by human RBC. This paper describes our findings.

EXPERIMENTAL

Chemicals

MMPR, MMPR-^{35} S, 6-methylthiopurine (NSC-20105, MMP), and 6-mercapto-9-β-D-ribofuranosylpurine (NSC-4911, MPR) were all supplied by the Drug Development Branch of the Cancer Chemotherapy National Service Center, National Cancer Institute. Dithioinosine disulfide [6,6-dithio*bis*(9-β-D-ribofuranosylpurine)] was supplied through the courtesy of Dr. J. J. Fox, of Sloan–Kettering Institute for Cancer Research. Tubercidin [4-amino-7-D-ribofuranosyl-7H-pyrrolo (2,3-*d*)pyrimidine] was a gift from Dr. G. S. Fonken of the Upjohn Company. Dipyridamole [2,6-*bis*(diethanolamino)-4,8-dipiperidino-pyrimido-(5,4-*d*)-pyrimidine] was generously provided by Geigy Pharmaceuticals. All other chemicals were procured from regular suppliers. Enzyme preparations were obtained from Sigma Chemical Company.

Blood

Either fresh heparinized blood collected by venipuncture from normal healthy volunteers or recently outdated whole blood (preserved in citric acid–dextrose) from the blood bank was used in this work.

Incubation and sample preparation

A solution of MMPR-^{35} S in normal saline (1 mg/ml, about 8×10^5 cpm/ml) was diluted with 9 vol. of whole blood so that the MMPR concentration was 100 μg per ml or 0·3 mM. This concentration was chosen to approximate the initial blood level of MMPR in a man of 70 kg who had been given a therapeutic dose of 350 mg per M^2, assuming the drug was evenly distributed exclusively in the blood. The blood was transferred into an Erlenmeyer flask and the flask was shaken at 100 cpm in a Dubnoff incubator at 37° for 60 min in ambient air. In time course studies, an aliquot of 2·0 ml of the blood was removed at intervals and analyzed (see below). In K_m and inhibition studies, incubation was terminated after 15 min by immersion in an ice bath. The initial concentration of MMPR in K_m studies varied from 0·05 to 0·3 mM in whole blood.

Immediately after incubation 2·0 ml of the blood was centrifuged at 10,000 rpm (about 9000 *g* in an International Equipment Co. high-speed centrifuge, model B20, equipped with an 874 rotor) for 10 min at 0°. The plasma was carefully separated from the packed RBC by aspiration; 0·2 ml of the plasma was directly counted as described below. The excess plasma and other cellular elements (buffy coat) were completely removed and discarded. An aliquot of 0·1 ml of packed RBC was lysed with 9·9 ml of distilled water, and 0·2 ml of the lysed cell suspension was used for radioactivity determination. RBC ghosts contributed little to the radioactivity and were therefore not removed. Also, it was not necessary to wash the packed RBC with normal saline before lysis because such a procedure did not significantly affect the counts.

Radioactivity determination

All plasma and lysed RBC samples (0·2 ml in all cases) were directly counted as a suspension in 11 ml of a counting solution as previously described.[5] Quenching was determined by channel ratios of automatic external standards. Relative counting efficiency was over 85 per cent.

Recovery of MMPR metabolite from RBC

Identification of the MMPR metabolite by thin-layer chromatography on cellulose MN 300 plates followed previously described procedures.[5] In addition, the metabolite was cleaved with either alkaline phosphatase (EC 3.1.3.1) of calf intestinal mucosa (Sigma type II) or 5'-nucleotidase (EC 3.1.3.5) of rattle snake (*Crotalus adamanteus*) venom (Sigma grade II) as described previously.[7] The hydrolytic product was compared chromatographically with authentic MMPR-^{35} S.

RESULTS

Uptake of MMPR-^{35} S by human RBC

The time course of a typical experiment is depicted in Fig. 1. The concentration

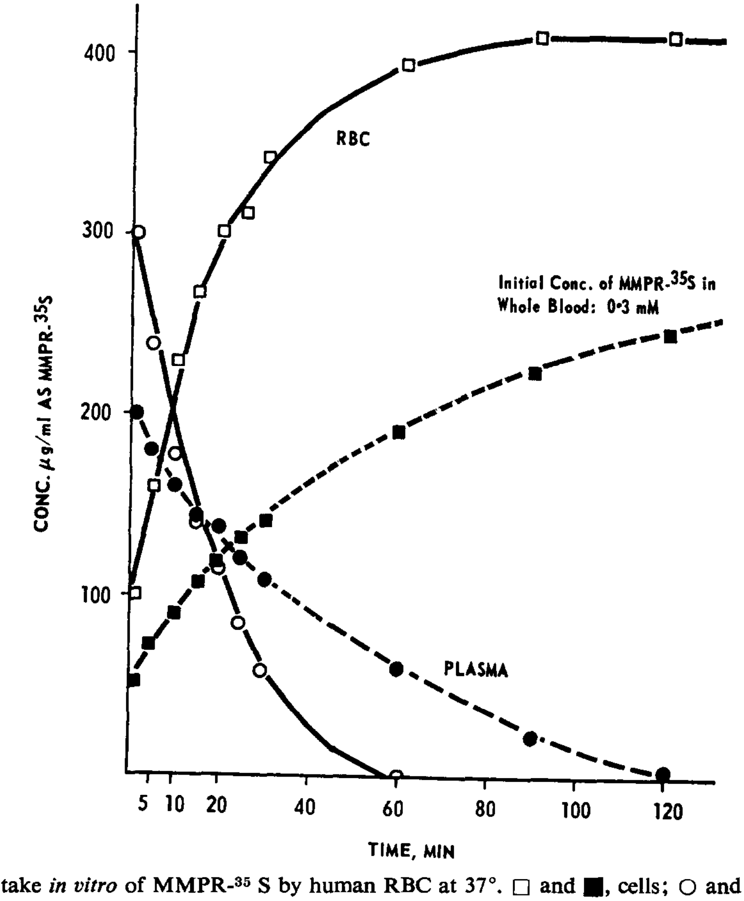

FIG. 1. Uptake *in vitro* of MMPR-^{35} S by human RBC at 37°. □ and ■, cells; ○ and ●, plasma Solid line, control; dotted line, after preloading with unlabeled MMPR. Initial concentration of MMPR-^{35} S in whole blood, 0·3 mM. Typical of five experiments.

is expressed in micrograms of MMPR-^{35} S per milliliter of intracellular water, after correction for extracellular water in the packed cell mass.[6] The initial rate of uptake was fast, almost linear with time for the first 15 min. Steady state was reached in 1 hr, at which time the RBC to plasma radioactivity ratio was 150 to 1 (average of ten experiments). The cells continued to take up radioactivity, although plasma concentration of MMPR-^{35} S had fallen below that in RBC in less than 10 min. After a 2-hr incubation, the cells showed no noticeable morphological changes.

An aliquot of the same batch of blood was incubated with unlabeled MMPR (concentration of MMPR in whole blood, 0·3 mM) at 37° for 30 min. The cells were separated from plasma, washed twice with an equal volume of normal saline, and resuspended in fresh plasma containing MMPR-^{35} S. The concentration of MMPR-^{35} S in the reconstituted blood was identical to that in the above experiment. The dotted lines in Fig. 1 illustrate the result of this experiment. The decrease in both extent and rate of the uptake is evident. When unlabeled MMPR was replaced with saline in the preloading, the results obtained differed very little from the experiment in which there was no preloading.

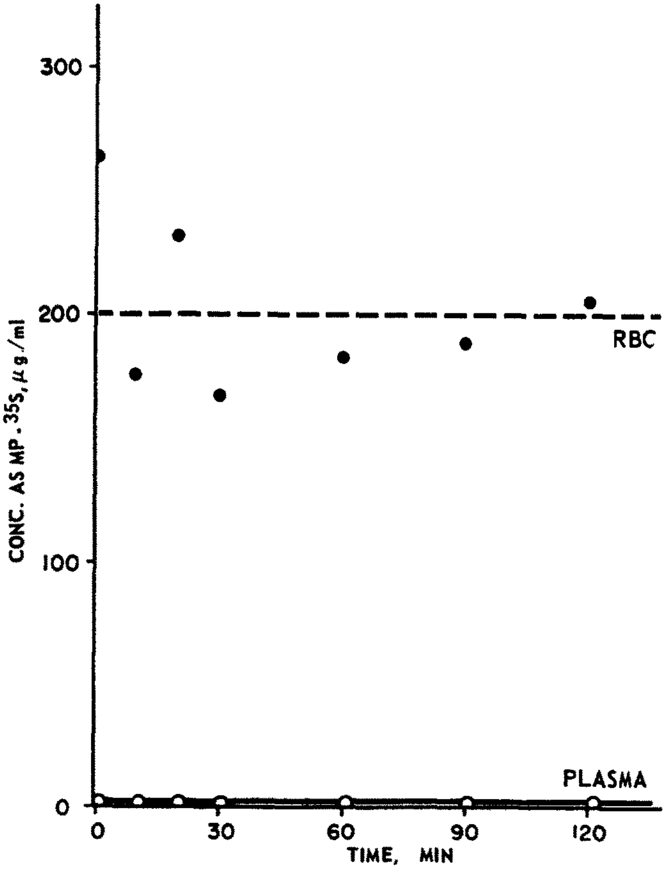

FIG. 2. Efflux of radioactivity from human RBC which had been preincubated with MMPR-^{35} S for 60 min. Typical of three experiments.

In another experiment, after incubation with MMPR-^{35}S for 60 min, the cells were separated from the plasma, washed twice with an equal volume of saline, and resuspended in fresh plasma. Incubation was resumed and continued for another 2 hr. Aliquots of blood were sampled at intervals. The cells and the plasma were separately counted as before. The efflux of radioactivity from the cells is negligible once the drug has gained entrance into the cells (Fig. 2).

Uptake of MP-^{35}S by human RBC

Similar experiments were performed with MP-^{35}S. The drug was dissolved in dilute sodium hydroxide and mixed with whole blood. The initial concentration of MP was 0·66 mM and that of sodium hydroxide was 10 mM. The addition of sodium hydroxide caused an increase of blood pH of less than 0·05. Steady state was attained in about 30 min, and the ratio of total radioactivity in RBC to that in plasma was 1 to 3. The concentrative transport seen with MMPR was not observed in these experiments (Fig. 3).

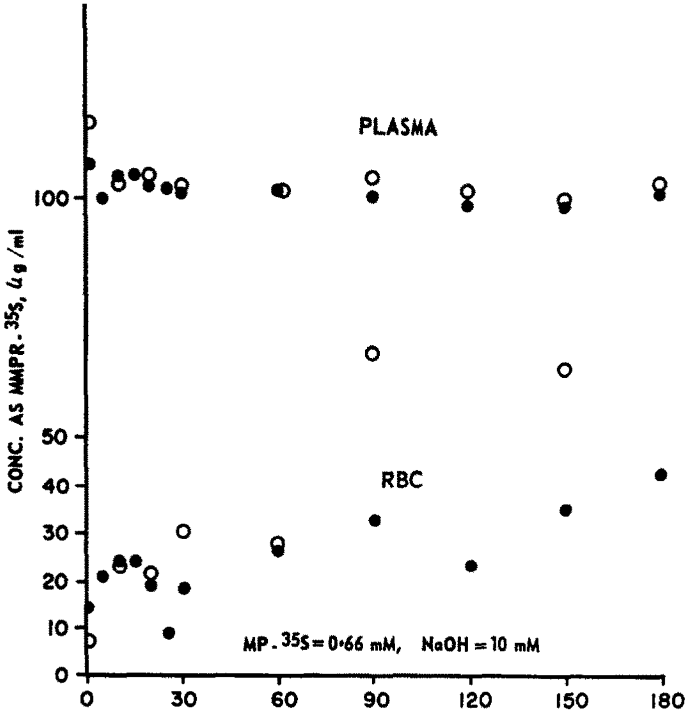

FIG. 3. Uptake *in vitro* of MP-^{35}S by human RBC at 37°. Two separate experiments are depicted.

Kinetics of MMPR uptake

Within an MMPR-^{35}S concentration range of 0·05 to 0·3 mM in whole blood, the uptake follows Michaelis–Menten kinetics. A typical double reciprocal plot of maximal velocity of the uptake against drug concentration[8] gives a straight line (Fig. 4). The method of least squares was employed to fit the experimental points.

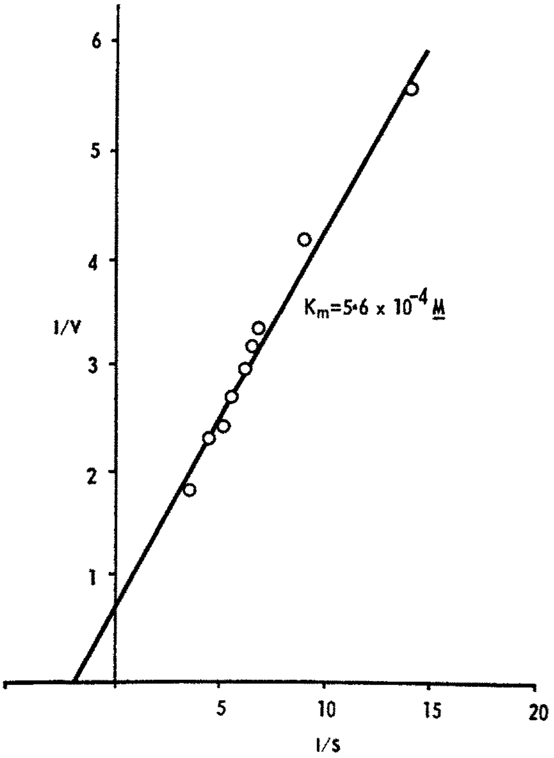

FIG. 4. Lineweaver–Burk plot in the uptake *in vitro* of MMPR-^{35}S by human RBC. *V*, in moles of radioactivity (as MMPR-^{35}S) taken up by 1 ml of packed RBC (corrected for extracellular water) in 15 min; *S*, concentration in moles/liter of MMPR-^{35}S in whole blood. Typical of twenty experiments.

No appreciable deviation from linearity was seen in experiments in which concentrations of MMPR higher than K_m were also used. The average value of the K_m of the uptake calculated graphically in 20 experiments is 0·91 mM, ranging from 0·13 to 2·59 mM at 37°. There is no consistent pattern of variation in K_m within a wide range of cell count (4×10^6 to 6×10^6 per mm^3) and hematocrit (40–55 per cent). Assuming that K_m is approximately the same as the equilibrium constant of the uptake, the corresponding value of the average free energy change at 37° is 4·32 kcal/M, ranging from 3·67 to 5·52 kcal/M.

Concentration dependence of the rate of uptake is further illustrated in Fig. 5 in which RBC radioactivity concentration (expressed as μg/ml of MMPR-^{35}S) attained in 15 min is plotted against plasma concentration. Below a plasma concentration of 5 μg/ml (0·015 mM), the ratio of intracellular to extracellular radioactivity (slope of the steep portion of the curve) has a constant value of 28. As the extracellular drug concentration exceeds 5 μg/ml, the ratio sharply declines. In another two experiments, the ratios are 53 and 54, and the critical extracellular drug concentration is the same. When the incubation was terminated at 60 min instead of at 15 min, a similar change in ratio was seen.

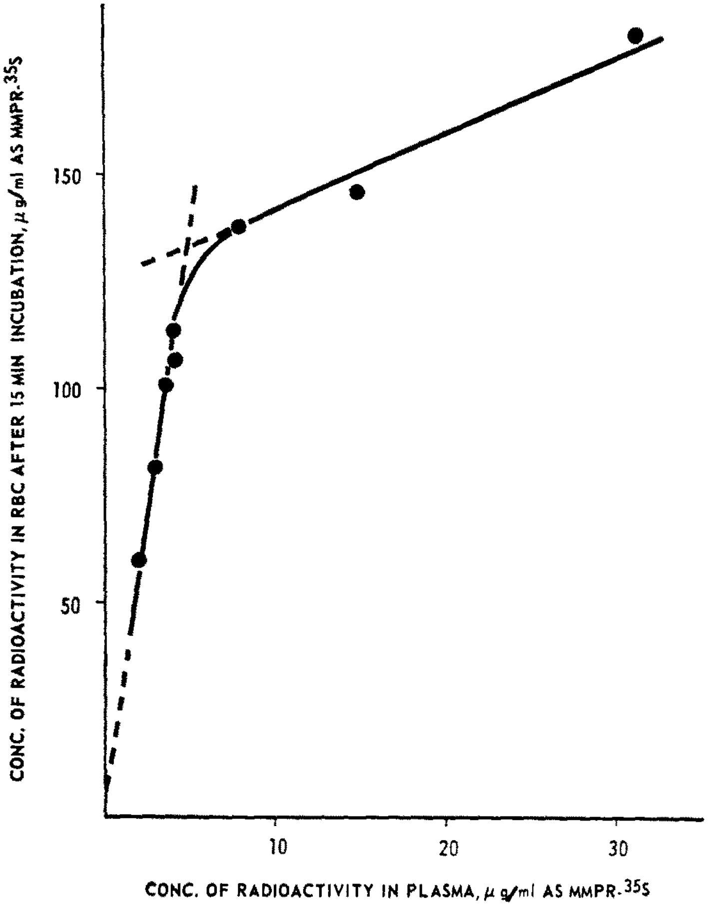

FIG. 5. Saturability of the uptake of MMPR-^{35} S by human RBC. Abscissa, plasma concentration of MMPR-^{35} S in μg/ml; ordinate, amount in μg MMPR-^{35} S taken up/ml of packed RBC in 15 min. Typical of five experiments.

Effect of temperature

The K_m and $V_{\max}$ of the uptake were measured at several temperatures with the same blood sample. A plot of log K_m against reciprocal absolute temperature[9] is shown in Fig. 6. A similar plot of log $V_{\max}$[10] is shown in Fig. 7.

The enthalpy of the uptake, ΔH, is calculated from Fig. 6 by the van't Hoff equation, assuming that K_m is approximately the same as the equilibrium constant.

$$\Delta H = -R \frac{\mathrm{d} \ln K_m}{\mathrm{d}\,(1/T)} \tag{1}$$

Similarly, the apparent activation energy, ΔE, and the temperature coefficient, Q_{10}, are estimated from Fig. 7 by:

$$\Delta E = -R \frac{\mathrm{d} \ln V_{\max}}{\mathrm{d}\,(1/T)} \tag{2}$$

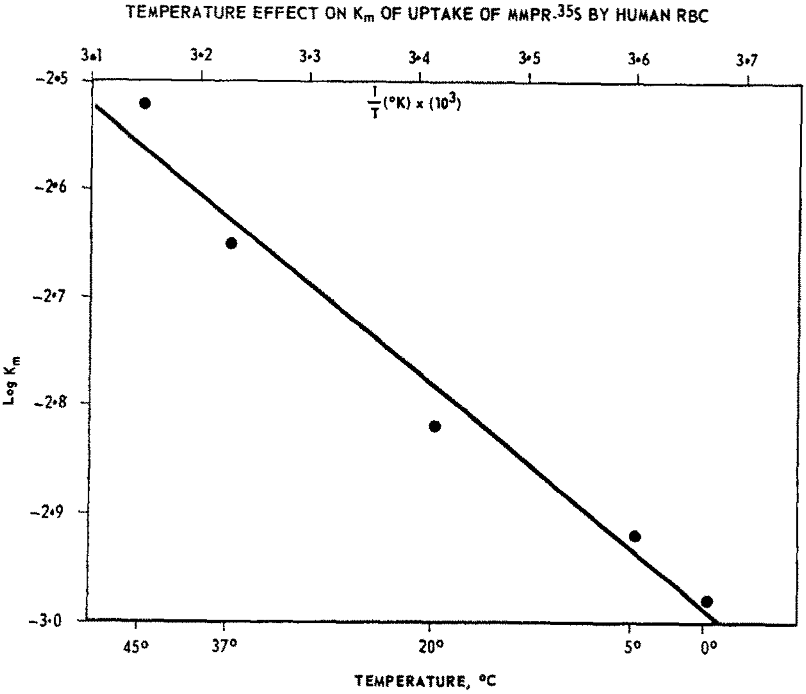

Fig. 6. Van't Hoff plot of log K_m vs. reciprocal absolute temperature. One of two experiments.

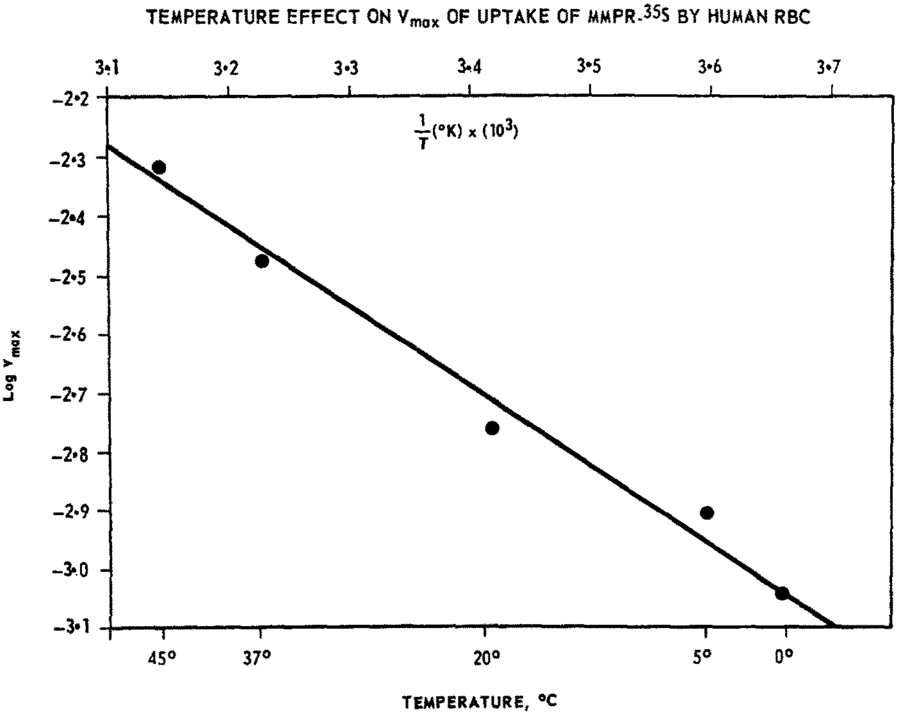

Fig. 7. Arrhenius plot of log V_{max} vs. reciprocal absolute temperature. One of two experiments.

In two experiments ΔH is estimated to be 3·3 and 3·8 kcal/M and ΔE is 6·1 and 6·7 kcal/M respectively. The corresponding values of Q_{10} are 1·41 and 1·45 (both between 27° and 37°).

Incubation under oxygen or nitrogen

The difference in uptake rates was not appreciable whether the incubation was carried out under air, oxygen (containing 5% carbon dioxide) or nitrogen.

Effects of analogs on MMPR uptake

Structural analogs were tested for their effect on the uptake at 37° (Table 1). The concentration of MMPR-^{35}S varied from 0·05 to 0·3 mM in these studies. The effects were highly dependent upon analog concentration and the blood sample used. The inhibitors were classified on the basis of double reciprocal plots. For example, at a concentration of 0·89 mM, adenosine is a competitive inhibitor (Fig. 8). Deoxyadenosine inhibits competitively at 1·9 mM, but noncompetitively at 0·95 mM (Fig. 9). Inosine is an uncompetitive inhibitor (Fig. 10). MP, at a concentration of 1·79 mM, approximately the therapeutic dose in combination therapy with MMPR, has no apparent influence on the uptake.

TABLE 1. ANALOG INHIBITION OF MMPR UPTAKE BY HUMAN RBC *IN VITRO**

Analog	Conc. (mM)	K_i (mM)
Competitive inhibitors		
Adenosine	0·89	2·54
6-Methylaminopurine ribonucleoside	0·95	2·10
Deoxyadenosine	1·90	7.84
6-Methylaminopurine deoxyribonucleoside	0·95	3·02
Deoxyinosine	0·95	3·40
Noncompetitive inhibitors		
Inosine	9·52	12·0
Deoxyadenosine	0·95	0·14
Uncompetitive inhibitor		
MPR	3·56	2·52
Noninhibitors		
MP	1·79	
MPR	1·50	
MMP	1·90	
Dithioinosine disulfide†	0·01	
AMP	0·95	
ADP	0·95	
Azaguanine	4·76	
D-Glucose	1·06	
D-Ribose	1·13	
Tubercidin†	0·95	
Dipyridamole†	0·95	
Caffeine	0·95	
Theobromine	0·95	

* All experiments had been repeated at least three times. Values of K_i are the mean of three experiments. For abbreviations, see text.

† See Discussion.

Effect of nonspecific inhibitors

With a few exceptions, most nonspecific inhibitors are without any effect. Table 2 represents a summary of our studies with nonspecific inhibitors.

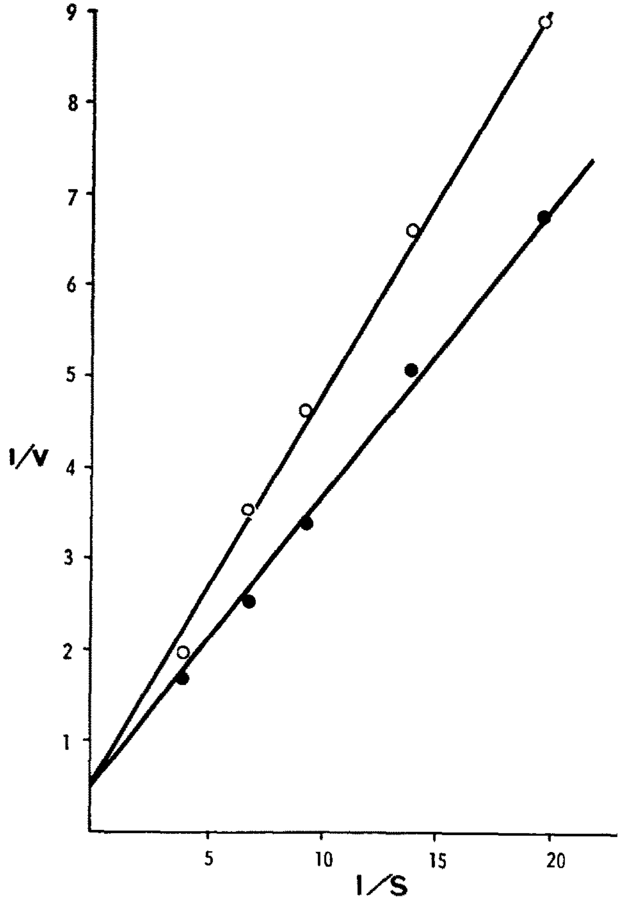

FIG. 8. Inhibition of adenosine (0·9 mM) of the uptake of MMPR-^{35}S by human RBC. *V* and *S* in same units as in Fig. 4. ●, MMPR alone; ○, MMPR and adenosine. Typical of seven experiments.

TABLE 2. EFFECT OF NONSPECIFIC INHIBITORS ON MMPR UPTAKE BY HUMAN RBC *IN VITRO**

Compound	Conc. (mM)
Inhibitors	
p-Chloromercuribenzoic acid (PCMB)	1·9
2,4-Dinitrofluorobenzene (DNFB)	0·19
Iodoacetic acid	0·95
Sodium azide	0·95
Uranyl chloride	0·95
Noninhibitors	
2,4-Dinitrophenol (DNP)	4·76
N-Ethylmaleimide (NEM)	0·95
Phloridzin	0·10
Phloretin	0·95
Ouabain	0·95
Sodium arsenate	0·95
Sodium cyanide	0·95
Sodium fluoride	0·95
Cupric sulfate	0·94
Zinc sulfate	0·94

* All experiments had been repeated at least 3 times. Concentrations of inhibitors are those that gave definite inhibition.

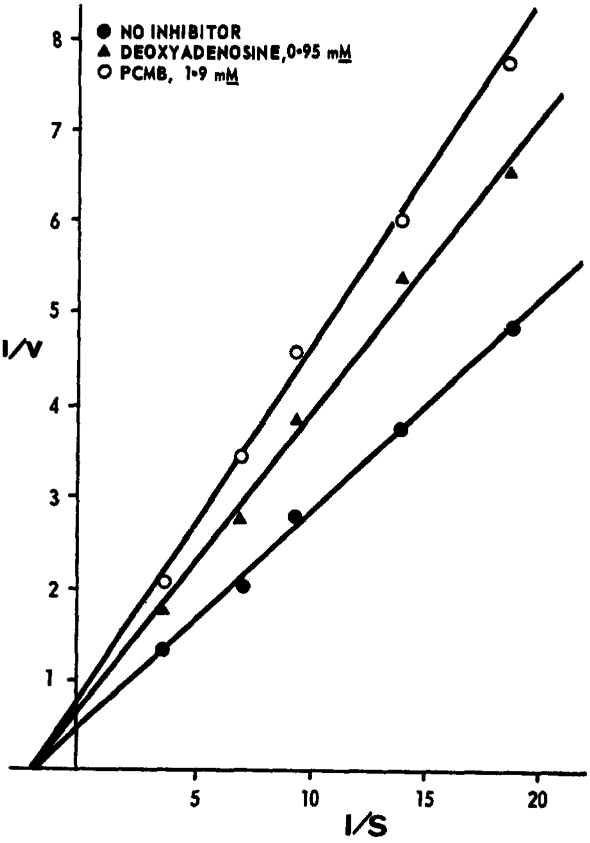

FIG. 9. Inhibition by deoxyadenosine and PCMB of the uptake of MMPR-^{35}S by human RBC. *V* and *S* in same units as in Fig. 4. Typical of five experiments.

Recovery and identification of the MMPR metabolite

All of the radioactivity in the cells was in the form of the 5′-monophosphate of MMPR.[5] The procedure for the recovery and identification of the MMPR metabolite from human RBC after incubation *in vitro* was the same as that employed in our clinical pharmacological work.[5] Further, upon enzymatic cleavage,[7] this metabolite gave a product which was chromatographically indistinguishable from authentic MMPR-^{35}S.

Enzymatic studies with adenosine kinase of human RBC

Adenosine kinase (EC 2.7.1.20) was prepared from human RBC by a published procedure.[7] Inhibition studies were performed with analogs of MMPR and also with nonspecific inhibitors, as described previously.[7] MPR, inosine, iodoacetic acid, 2,4-dinitrophenol, ouabain, phloridzin and phloretin, at concentrations of 0·06 and 6 mM, were without effect on the enzyme. Some of the results are presented in Table 3

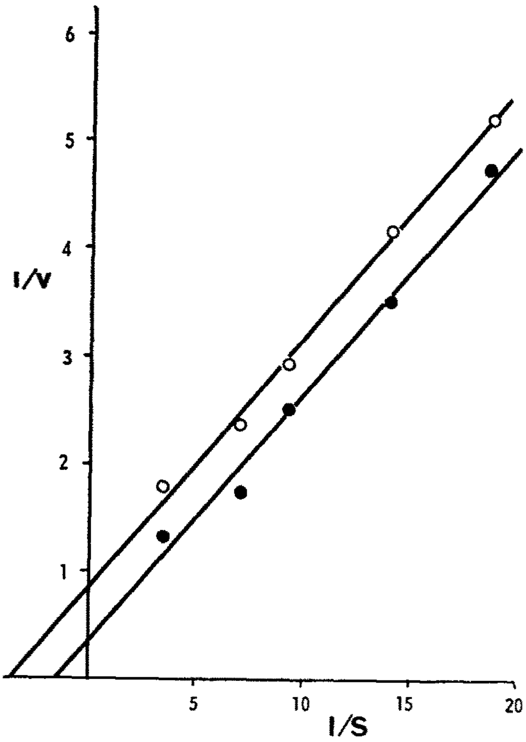

FIG. 10. Inhibition by MPR (3·6 mM) of the uptake of MMPR-^{35}S by human RBC. *V* and *S* in same units as in Fig. 4. ●, MMPR alone; ○ MMPR and MPR. Typical of five experiments.

TABLE 3. EFFECT OF MMPR ANALOGS ON THE PHOSPHORYLATION OF MMPR BY ADENOSINE KINASE OF HUMAN RBC*

Compound	Conc. (mM)	Effect
Adenosine	6	Complete inhibition
	0·6	Complete inhibition
	0·06	Complete inhibition
6-Methylaminopurine riboside	0·6	No effect
	0·06	No effect
6-Methylaminopurine deoxyribonucleoside	6	87% Inhibition
	0·6	50% Inhibition
	0·06	10% Inhibition
Dipyridamole	6	15% Inhibition
	0·6	No effect
	0·06	No effect

* All experiments had been repeated at least three times. Initial concentration of MMPR-^{35}S, 0·6 mM.

DISCUSSION

In many respects the uptake of MMPR by human RBC *in vitro* appears to be an active process. However, the complete intracellular biotransformation of MMPR to the phosphorylated derivative, the 5′-monophosphate, could explain the intracellular accumulation and persistence of radioactivity without invoking an active mechanism. The observed K_m values, saturability and temperature effect therefore apply to the combined process of uptake and intracellular phosphorylation.

MP, the parent compound, does not exhibit similar transport properties. Moreover, at steady state, the RBC to plasma total radioactivity ratio is 1 to 3 in favor of plasma (Fig. 3). MPR, the S-demethylated analog of MMPR, is also taken up by human RBC concentratively. Nevertheless, the mechanism is quite different.* In addition, the steady state ratio of RBC to plasma radioactivity is only 4 to 1.

Structural specificity of the uptake is further demonstrated by inhibition studies with MMPR analogs (Table 1). Either the transport carrier site or the enzyme adenosine kinase can be affected. The effect of these analogs is highly concentration dependent. As an example, although not inhibitory at 1·5 mM, MPR is an uncompetitive inhibitor at 3·56 mM (Fig. 10). In contrast, MP at concentrations up to 1·79 mM exerts no influence on the uptake, in agreement with our clinical pharmacological findings.[5] Likewise, MMP is without effect on the uptake. Dithioinosine disulfide, a potent inhibitor of adenosine kinase from human H.Ep. No. 2 cells,[11] shows no inhibition of the uptake. 8-Azaguanine, an inhibitor of adenosine deaminase,[12] fails to enhance the inhibition due to adenosine at up to 5-fold the concentration of the latter. Caffeine, known to inhibit inosine transport and adenosine incorporation into phosphorylated acid-soluble intermediates and nucleic acids of *Escherichia coli*,[13] does not interfere with MMPR uptake by the RBC.

The influence of tubercidin and dipyridamole on MMPR uptake has been examined because tubercidin, like MMPR, is also preferentially taken up by the human RBC,[14] and dipyridamole decreases the permeability of the RBC for adenosine.[15] Tubercidin initially stimulates MMPR uptake, but 15 min after incubation the effect is reversed; it inhibits the uptake. Dipyridamole resembles tubercidin, but the critical time for the reversal is between 25 and 40 min.

With the exception of *p*-chloromercuribenzoic acid (PCMB), iodoacetic acid, sodium azide and uranyl chloride, most nonspecific inhibitors are without effect on the uptake (Table 2). Uncouplers of oxidative phosphorylation, notably DNP, sodium azide and sodium arsenate, do not inhibit the uptake, suggesting that oxidative phosphorylation is not the energy source of the uptake. Also, this energy is not derived from glycolysis, since sodium fluoride is not inhibitory.

The temperature coefficient (Q_{10}) of the uptake in the range 27°–37° is about 1·4 (Fig. 7), quite comparable to the Q_{10} (1·21) of free diffusion of potassium in solution in the range of 28°–38°.[16] A value of 2·21 has been reported for the Q_{10} of uric acid transport, presumably by facilitated diffusion, in the human RBC.[17] A high Q_{10} has frequently been associated with an active process and, conversely, a low Q_{10} with a passive one. To deduce transport mechanism on the strength of temperature effect alone can be misleading, since high Q_{10} values are known even for some passive fluxes.[18, 19] Passive diffusion with a high Q_{10} value has not been reported.

The uptake of MMPR by the RBC may not merely be a manifestation of cellular

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* Ti Li Loo, unpublished work.

adenosine kinase activity. 6-Methylaminopurine riboside (Tables 1, 3) and deoxyadenosine[20] interfere with the uptake even though they are not inhibitors of adenosine kinase. Furthermore, as a fairly large molecule with a hydrophilic ribose moiety attached to a purine base, MMPR apparently enters the RBC with surprising ease. The human RBC is highly permeable to adenine, hypoxanthine (but not MP) and their ribonucleosides, adenosine and inosine, with an even distribution between cells and medium.[21] Further studies of hypoxanthine reveal that its uptake by the human RBC involves a saturable carrier system in addition to passive diffusion,[22] reminiscent of our saturation studies (Fig. 5). The purine ribonucleosides can be metabolized under conditions in which the RBC cannot efficiently utilize glucose; for this reason, they are energetically more favourable than glucose.[23] Possibly, therefore, the transport of purines and their nucleosides into the RBC is carrier mediated.[24, 25] Nucleoside transport in *E. coli* is postulated to possess membrane sites carrying ribonucleoside permease.[13] That MMPR is implicated in purine nucleoside transport across the RBC membrane has been suggested by a number of authors.[25, 26]

Membrane permeation accompanied by simultaneous biotransformation of the permeant has been well documented. For instance, after penetration into the RBC, adenine is converted into nucleotides to which the RBC membrane is impermeable.[21, 27, 28] Incubation of the human RBC with radioactive hypoxanthine, inosine or adenosine results in labeling of the hypoxanthine portion of IMP.[28] The uptake of nicotinic acid and nicotinamide by the rat RBC is reported to consist of diffusion and enzymatic conversion to nucleotides.[29] Finally, phosphorylation has also been reported to accompany uphill transport of thiamine by everted rat intestinal sacs,[30] quite similar to our findings except that DNP and sodium fluoride greatly reduce both net transport and phosphorylation of thiamine.

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