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ADENINE NUCLEOTIDE METABOLISM OF BLOOD PLATELETS

VIII. TRANSPORT OF ADENINE INTO HUMAN PLATELETS

JAN J. SIXMA*, HOLM HOLMSEN and ANNEMIEKE C. M. TRIESCHNIGG*

Institute for Thrombosis Research, Rikshospitalet, Oslo 1 (Norway)

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SUMMARY

Adenine uptake into human blood platelets is a carrier-mediated process with a K_m of 159±21 nM and a V of 100±10 pmoles/min per 10^9 platelets (in citrated platelet-rich plasma). The Q_{10} was 2.53±0.22. A pH optimum was found at 7.5. Washing of the platelets increased the K_m to 453±33 nM and V to 397±38 pmoles/min per 10^9 platelets. The change in shape induced in platelets by ADP was accompanied by an increase in V (2 times) and K_m (1.5 times).

Guanine (K_i 50 μM), hypoxanthine (K_i 390 μM), adenine-*N'*-oxide (K_i 40 μM), adenosine (K_i 100 μM), RA 233 (K_i 75 μM) and papaverine (K_i 15 μM) acted as competitive inhibitors. Adenosine at low concentrations, and prostaglandin E_1 gave inhibition at only high adenine levels. A similar inhibition was obtained with 2-deoxy-D-glucose. Sulfhydryl-group inhibitors, pyrimidines and ouabain had no effect.

INTRODUCTION

Adenine transport across the cell membrane is a carrier-mediated process in bacteria[1–5], yeast [6,7], rabbit choroid plexus[8], human red blood cells[9,10] and rabbit polymorphonuclear leukocytes[11]. Holmsen and Rozenberg[12] have shown that human platelets take up adenine by a saturable process, which is inhibited by adenosine. Adenosine is also a powerful inhibitor of platelet aggregation and its inhibitory action is enhanced by dipyridamole which blocks adenosine uptake. Rozenberg *et al.*[13] showed that the inhibitory effect of adenosine on adenine uptake was abolished under these conditions. No direct effect of dipyridamole on adenine transport was observed.

The present study deals with the kinetics of the transport of adenine into human blood platelets and its inhibition by adenosine.

METHODS

[G-^{3}H]inulin (217 mCi/g) and [G-^{3}H]adenine (6.7 Ci/mmole) were purchased from New England Nuclear (Boston). High-voltage paper electrophoresis[14,15]

* On leave of absence from Division of Haemostasis, Department of Internal Medicine, University Hospital Utrecht, The Netherlands.

showed that at least 93% of the radioactivity was adenine. All purine and pyrimidine bases and nucleosides were obtained from Sigma Chemical Co. (St. Louis). All other chemicals were "pure" or "reagent" grade.

Blood from donors who used no drugs, was collected into citrate (1 volume 0.11 M disodium citrate to 9 volumes blood) or EDTA (1 volume 0.027 M EDTA in 0.12 M NaCl to 9 vol. blood) and centrifuged at $g_{max}=180\times g$ for 10 min at room temperature. The supernatant platelet-rich plasma was kept at room temperature until use. Platelet suspensions were prepared by centrifugation of EDTA platelet-rich plasma at $g_{max}=1000\times g$ for 10 min. The platelet pellet was resuspended in the medium described by Gaintner *et al.*[16] (0.103 M NaCl, 0.04 M NaH_2PO_4, 0.0047 M KH_2PO_4, 0.005 M glucose, 0.005 M Na_2EDTA, adjusted to pH 7.6 ± 0.1 with 0.04 M NaOH) centrifuged again and resuspended in 80% of the original volume of this medium. All these procedures were carried out at room temperature.

Adenine is not metabolized in plasma or in the extra cellular phase of platelet suspension[10]. Adenine uptake was therefore measured as the rate of disappearance of total extracellular radioactivity during incubation with [^{3}H]adenine. The validity of this procedure was established by comparing with adenine disappearance from the supernatant as measured after separation with high voltage paper electrophoresis[14,15]. In most of the experiments described, adenine uptake was measured in the following way: Portions (950 μl) of platelet-rich plasma or platelet suspensions were warmed for 5 min at 37 °C. [^{3}H]Adenine (50 μl) was added and 50 μl of the resulting mixture was transferred directly to 10 ml scintillation fluid (5 g diphenyloxazole, 100 g naphthalene per l l dioxane) for determination of zero time radioactivity. After incubation for a recorded period of time two portions (250 μl) were distributed into tubes kept in an ice bath. The incubation mixture was not shaken during incubation as separate experiments had shown this had no effect on adenine uptake. The portions were centrifuged (30 s at $g_{max}=20\,000\times g$ at room temperature in a microcentrifuge (Ole Dick, Hvidovre, Denmark) and 50 μl of the supernatant were used for determination of the radioactivity. The cpm/pmole of [^{3}H]adenine was calculated from the zero time values for each set of samples. The uptake of adenine was calculated as pmole/min per ml and corrected for platelet number. The coefficient of variation of adenine uptake was estimated by 12-fold determination. A value of 9.7% was obtained at 75 nM and of 19.6% at 900 nM adenine. The high coefficient of variation at the high adenine concentration was caused by the uptake being only a small part of the total radioactivity in the supernatant. Uptake at higher adenine concentrations was therefore studied with another method (for description Fig. 2).

Platelet counts were carried out for every platelet-rich plasma or platelet suspension with a counting chamber method.

RESULTS

Adenine uptake in citrate platelet-rich plasma at 37 °C was linear with time for 40 min at 1 μM, for 10 min at 0.3 μM, for 7 min at 0.15 μM and for 4 min at 50 nM (Fig. 1). The process was saturable, and followed Michaelis–Menten kinetics with an apparent K_m or 159 ± 21 (S.E.) nM and a V of 100 ± 10 (S.E.) pmoles/min per 10^9 platelets (21 experiments). The V increased linearly with platelet number.

The uptake was temperature dependent with a $Q_{10}=2.53\pm0.22$ (S.E.) (mean value from 6 Arrhenius plots with duplicate determinations at 6 temperatures, 0° and 25–45 °C, adenine 0.9 μM). All the experiments described above were carried out within 1 h after blood collection. Storage of EDTA platelet-rich plasma produced an initial strong stimulation in uptake to 2–3 times initial values 90–120 min after blood collection followed by a decrease to low levels 180 min after blood collection.

Adenine was taken up by washed platelets with an apparent K_m and a V 3–4 times as high as in platelet-rich plasma ($K_m=453\pm33$ nM (S.E.): $V=397\pm38$ (S.E.) pmoles/min per 10^9 platelets: 16 experiments (Fig. 2). The V increased linearly with platelet number. Addition of increasing volumes of platelet-poor plasma instead of buffer did not influence the uptake rate. Adenine uptake in platelet suspension was not influenced by storage between 2 and 4 h after blood collection. The effect of washing might (at least in part) be explained by the storage phenomenon as washing was finished about 2 h after blood collection.

The influence of phosphate ions and/or glucose was studied by comparing adenine uptake into washed platelets which were resuspended in either the phos-

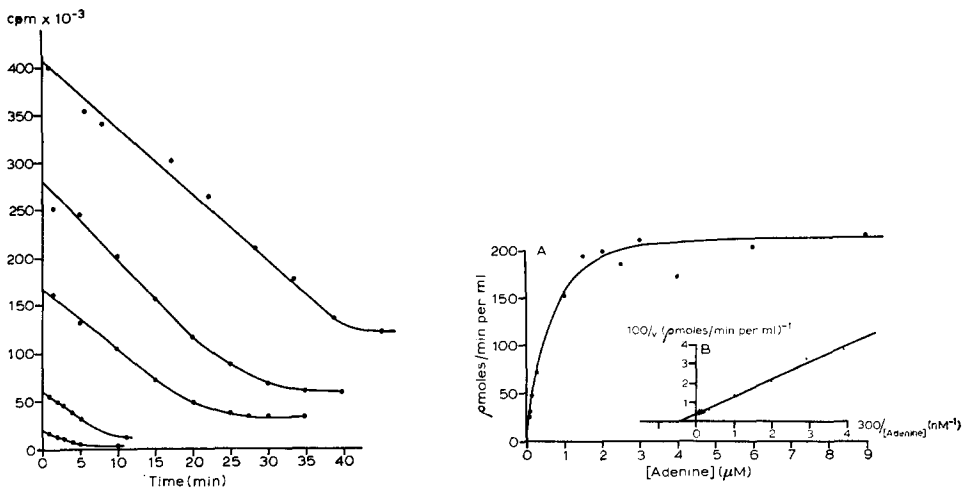

Fig. 1. Time dependence of adenine uptake. 500 μl [^{3}H]adenine in 0.154 M NaCl was added to 4.5 ml citrate platelet-rich plasma previously preincubated for 5 min at 37 °C. 200- and 50-μl aliquots were removed at the indicated time points, centrifuged and 50 μl of the supernatant was counted. The curves shown are for 1 μM, 0.7 μM, 0.4 μM, 0.15 μM and 50 nM, respectively. The experiments were performed using plasma from different donors and this explains the differences in velocity at saturating concentrations.

Fig. 2. Concentration dependence of adenine uptake. (A) Adenine uptake in washed platelets was studied after incubation as described in Methods by centrifugation of 250-μl samples for 2 min in an Eppendorf centrifuge 3200 at $g_{max}=10000\times g$ at room temperature. The supernatant was discarded and the tube was wiped dry with a cotton wool tipped stick. The pellet was washed twice with 250 μl of washing medium and resuspended in 250 μl of 0.5% Triton X-100 in 0.154 M NaCl and frozen–thawed twice. Duplicate samples of 50 μl were counted for radioactivity. Correction was made for the amount of trapped medium by measuring in triplicate parallel experiments. The [^{3}H]inulin trapped was found to be 0.36% of the medium. Platelet number $5.8\cdot10^8$/ml. (B) Double-reciprocal plot of the same data as in A (V 244 pmoles/min per ml; K_m 670 nM).

phate buffered medium used for washing (see Methods) or in Tris-buffered saline (135 mM NaCl, 12 mM Tris–HCl, 1.54 mM Na_2-EDTA (pH 7.5)). No difference was observed. Uptake of adenine in washed platelets resuspended in 0.3 M sucrose was much decreased when compared with that obtained in Tris-buffered saline.

Platelets change from a discoid to a spherical shape during aggregation. Rapid shape change without aggregation can be produced by addition of ADP to platelets in the presence of EDTA[17]. Transport of adenine into platelets in plasma was strongly stimulated by ADP in presence of EDTA with a marked increase in V and also increase in apparent K_m. A similar experiment with washed platelets gave the same results (Fig. 3).

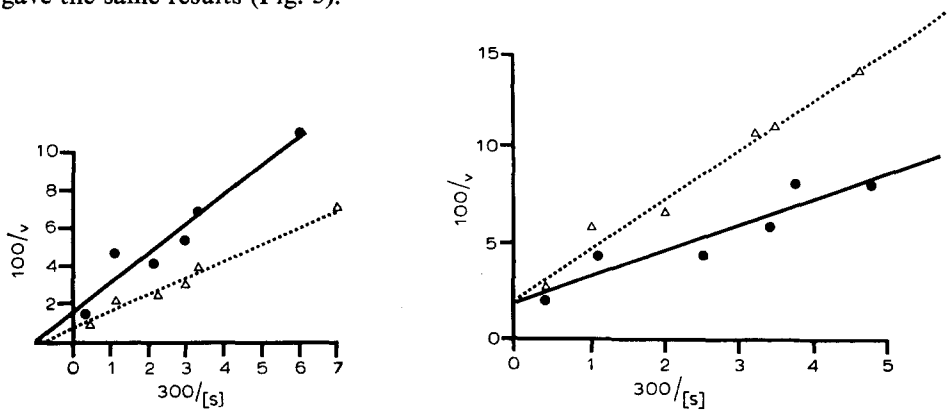

Fig. 3. Influence of ADP on adenine uptake by washed platelets. A suspension of washed platelets containing $1.6 \cdot 10^8$ platelets/ml was shaken in the presence or absence of 2.5 μM ADP for 30 s at 37 °C before addition of [^{3}H]-adenine. △.....△, ADP addition (V 97 pmoles/min per ml, K_m 536 nM); ●—●, control (V 46 pmoles/min per ml; K_m 379 nM).

Fig. 4. Influence of hypoxanthine on adenine uptake. Double reciprocal plot of velocity in pmoles/min per ml obtained in citrate platelet-rich plasma ($2.9 \cdot 10^8$ platelets/ml) *vs* adenine concentration in nM. △.....△, in the presence of 1 mM hypoxanthine (V 47 pmoles/min per ml; K_m 365 nM); ●—●, control (V 51 pmoles/min per ml; K_m 203 nM).

Adenine-N'-oxide, hypoxanthine and guanine were weak competitive inhibitors (Fig. 4). K_i values are given in Table I. Thymine and uracil (2 mM) tested at two adenine concentrations (900 and 75 nM) had no effect.

Adenosine at relatively low concentration (<20 μM) inhibited adenine uptake at high adenine concentrations (Fig. 5), thus resembling "substrate inhibition". Adenosine at high concentrations (>100 μM) acted as competitive inhibitor (Table I). Inosine (2 mM) was without effect.

2-Deoxy-D-glucose inhibited adenine uptake especially at high adenine concentrations. Antimycin A had no effect alone but enhanced the effect of 2-deoxy-D-glucose (Fig. 6). Prostaglandin E_1, which is a powerful activator of adenylate cyclase activity in platelets[18], produced a similar type of inhibition (Fig. 7).

The effect of various agents on adenine uptake are summarized in Table II. Bromomercurihydroxy propane, N-ethylmaleimide (especially after prolonged incubation), and 2,4-dinitrofluorobenzoate were inhibitory. Some inhibition was observed with iodoacetamide and N-acetylimidazole at high concentrations of adenine (0.9 μM).

TABLE I

K_i VALUES FOR COMPETITIVE INHIBITORS

All inhibitors were dissolved in saline and compared with control experiments with saline alone (50 μl to 0.9 ml platelet suspension with about $3 \cdot 10^8$ platelets/ml). 0.8 mM guanine was dissolved in 20 mM NaOH. Parallel experiments were here carried out with 20 mM NaOH alone. Figures in parentheses give number of experiments. K_i was calculated with the help of the formula $K_i = I/(K_p/K_m - 1)$ in which I is the inhibitor concentration, K_m the apparent Michaelis constant without inhibitor and K_p the apparent Michaelis constant in the presence of the inhibitor.

Inhibitor	K_i (μM)
Adenine-*N'*-oxide	40 (2)
Hypoxanthine	390 (3)
Guanine	50 (3)
Adenosine	100 (2)
Papaverine	15 (2)
RA 233	75 (2)

TABLE II

EFFECT OF INHIBITORS

The various substances were added in 50-μl volumes to 0.9 ml platelet suspension (about $3 \cdot 10^8$ platelets/ml) immediately followed by 50 μl [^{3}H]adenine. An exception was *N*-ethylmaleimide which was preincubated with the platelet suspension. Its reaction was stopped by adding equimolar dithiothreitol. Results are mean values obtained from two duplicate experiments. Results are expressed as percentages of saline controls. All substances were dissolved in 0.154 M NaCl with the following exceptions: *p*-hydroxymercuribenzoate was dissolved in 0.8 M NaOH. The pH was adjusted to 8.4 with 0.8 M HCl. The solution was diluted to 1 mM with distilled water and further to 200 μM with 0.154 M NaCl; 2,4-dinitrofluorobenzene was freshly prepared by dissolving in ethanol followed by 20 times dilution with 0.154 M NaCl to give a 4.8 mM solution.

Inhibitor	*Function or group affected*	*Percentage*	
		900 nM adenine	*75 nM adenine*
Ouabain, 100 μM	Na^+, K^+ pump	116	119
Mercaptoethanol, 1 mM	S–S	73	89
Iodoacetamide, 1 mM	–SH	43	72
Parahydroxymercuribenzoate, 10 μM	–SH	89	109
N-Ethylmaleimide, 1 mM, 3 min	–SH	56	91
N-Ethylmaleimide, 1 mM, 30 min		23	40
Bromomercurihydroxy propane, 1 mM	–SH	21	46
2,4-Dinitrofluorobenzene, 240 μM	NH_2	21	38
N-Acetyl imidazole, 10 mM, 30 min preincubation	Tyrosyl, $-NH_2$	49	82
Succinic acid anhydride, 10 mM	$-NH_2$	80	87
Atractyloside, 1 mM	Adenine nucleotide translocation	61	85

Dipyridamole, RA 233, RA 433, VK 744, VK 774 and papaverine were inhibitors of adenine uptake. Papaverine and RA 233 were investigated in more detail. Both were competitive inhibitors, with K_i values of 15 and 76 μM respectively. Dipyridamole, RA 233, VK 744 and papaverine produced 50% inhibition at 100 nM adenine at concentrations of, respectively: 0.25 mM, 0.7 mM, >10 mM and 50 μM.

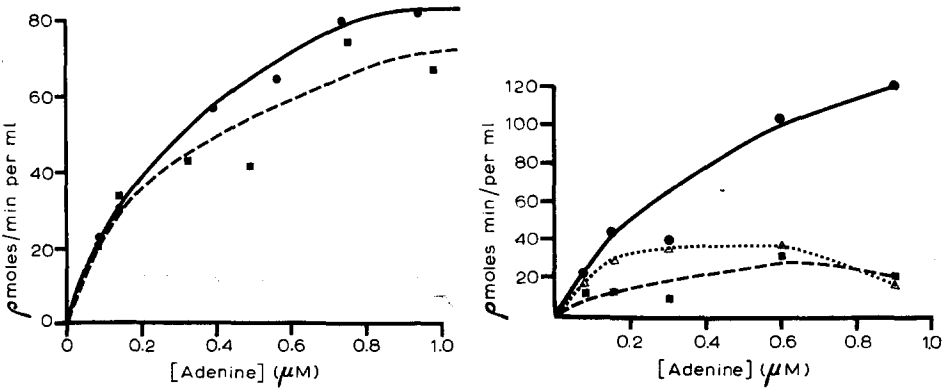

Fig. 5. Influence of adenosine on adenine uptake. Plot of adenine uptake in a suspension of washed platelets ($1.9 \cdot 10^8$ platelets/ml). ■—■, in the presence of 5 μM adenosine; ●—●, control.

Fig. 6. Influence of metabolic inhibitors on adenine uptake. A suspension of washed platelets ($2.5 \cdot 10^8$ platelets/ml) was preincubated 5 min at 37 °C with 7 mM 2-deoxy-D-glucose, or with 7 mM deoxyglucose together with 250 ng/ml antimycin A (final concn) added in 5 μl 96% ethanol just before adenine addition. Controls were incubated with 0.154 M NaCl. Control experiments showed that an ethanol concentration of below 0.5% as used here, had no influence upon uptake. △—△, deoxy-D-glucose; ■—■, deoxy-D-glucose+antimycin A; ●—●, control.

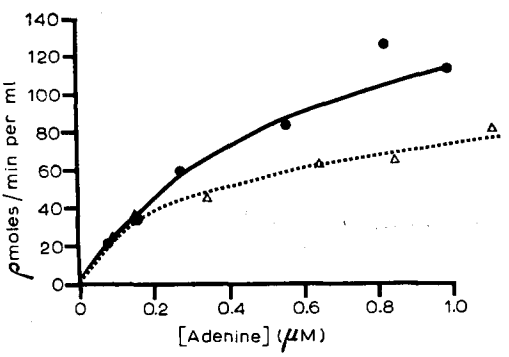

Fig. 7. Influence of prostaglandin E_1 on adenine uptake. A suspension of washed platelets ($2.1 \cdot 10^8$ platelets/ml) was preincubated for 5 min at 37 °C with 10 μM prostaglandin E_1 added in 5 μl methanol before addition of adenine. Control experiments showed that 0.5% methanol had no influence on uptake. △.....△, prostaglandin E_1; ●—●, control.

DISCUSSION

Adenine transport into human platelets can be considered unidirectional as adenine is immediately converted to adenine nucleotides on entering the cell[12].

TABLE III

CHARACTERISTICS OF ADENINE TRANSPORT FROM THE LITERATURE

Cell type	*Type of transport*	K_m	*Competitive inhibitors and K_i values*
Pseudomonas aeruginosa[1]	No transport		
Salmonella typhimurium[2]	Carrier mediated		Diaminopurine
Escherichia coli[3–5]	Carrier mediated	15 μM	
Saccharomyces pombe[6]	Carrier mediated	2.5 μM	Hypoxanthine (9 μM), guanine (34 μM) and diaminopurine (1.3 μM)
Rabbit chorioid plexus[8]	Carrier mediated		
Rabbit polymorphonuclear leucocytes[11]	Carrier mediated	7 μM	Hypoxanthine (50% inhibition at 1 mM), xanthine (40%
	Two systems	100 mM	inhibition at 5 mM), guanine (20% inhibition at 125 μM)
Human red blood cells[9,10]	Carrier mediated		Hypoxanthine, adenine, guanine uric acid
Human blood platelets[12]	Carrier mediated		
Human blood platelets (this paper)	Carrier mediated	0.16 μM	Hypoxanthine (390 μM), guanine (50 μM), adenine-N'-oxide (40 μM) and adenosine (100 μM)

The transport is carrier mediated, just as has been shown for other cells, and shows a high affinity and specificity for adenine. Comparison with other cells (Table III) shows that the affinity for adenine is much higher in platelets, whereas only polymorphonuclear leukocytes showed a similar specificity. This high affinity and specificity for adenine may have a bearing on the important function of adenine nucleotides in platelet functions[19] and the lack of *de novo* synthesis[10].

Both K_m and V for adenine transport into platelets are apparently influenced by the condition of the membrane: Storing and washing of platelets, and ADP, which induces a change in shape, increased both uptake parameters. The increased transport rate concomitant with shape change probably explains the increased uptake taking place with platelet aggregation[20].

Adenosine (below 20 μM) only affected adenine uptake at adenine levels higher than 200 nM. This inhibition pattern might be explained by assuming that adenosine competes with adenine for ATP necessary for incorporation of both precursors into nucleotides[12,21]. When the level of ATP is insufficient for the formation of enough phosphoribosyl pyrophosphate to incorporate adenine, a backflow may arise which immediately may result in an apparent decrease in uptake. This view is supported by the similar kinetics of inhibition by 2-deoxy-D-glucose and prostaglandin E_1. 2-Deoxy-D-glucose reduces the ATP level directly by consumption in formation of deoxyglucose 6-phosphate and indirectly by suppressing ATP resynthesis though blocking of the glycolytic flux[22,23].

Prostaglandin E_1 is a powerful stimulator of adenylate cyclase[18] and thus

protein kinase[24], which both consume ATP. Interestingly, the inhibitory action of prostaglandin E_1 on platelet functions has also been explained by competition for ATP between adenylate cyclase and ATP-requiring steps in platelet function[25,26].

Most platelet functions are inhibited by metabolic inhibitors, but a powerful inhibition is only obtained by a combination of a glycolytic and respiration inhibitor[27]. Adenine uptake is not influenced by antimycin A, but is strongly inhibited by 2-deoxy-D-glucose alone. This suggests that deoxyglucose is phosphorylated by the same (membrane) pool of ATP which is involved in adenine uptake. Our results suggest that energy might not only be required for incorporation of adenine into nucleotides but also for transport itself, as the transport rate is also decreased at low adenine concentrations.

Adenine phosphoribosyltransferase (EC 2.4.2.7) is probably directly involved in translocation of adenine across the membrane in *Escherichia coli*[3–5], but as far as we know no report on the existence of such a system in mammalian cells has been published. Such a mechanism might, however, explain the inhibition obtained with 2-deoxy-D-glucose. The Michaelis constant of platelet lysate adenine phosphoribosyltransferase for adenine (5.2 μM)[12] is about 10 times higher than that observed here for transport, but embedding of an enzyme within a membrane in an ordered manner might well increase its affinity for a substrate.

Various agents inhibited adenine uptake, but so far we have been unable to draw conclusions as to the nature of possible functional groups. The variable effect of different sulfhydryl-group inhibitors, as well as the time-dependent increase in inhibition with N-ethylmaleimide suggests that sulfhydryl groups are not directly involved but may act indirectly (*e. g.* by inhibiting glycolysis). This is supported by observations showing a lack of inhibition on uptake in isolated platelet membranes (Sixma, J. J., French, P. C. and Holmsen, H., unpublished). Exactly the same argument can be applied in the case of *N*-acetylimidazole which is more effective after preincubation and has no effect on uptake by isolated membranes (Sixma, J. J., French, P. C. and Holmsen, H., unpublished). The inhibition by 2,4-dinitrofluorobenzene may be caused by interaction with the carrier, but this agent has broad specificity.

The competitive inhibition by papaverine and pyrimidopyrimidine deserves special attention. The discrepancy with the results of Rozenberg *et al.*[13] who found no inhibition with dipyridamole is explained by the high adenine concentration they used, overcoming the action of dipyridamole. The reason for such a competitive inhibition is not clear as the molecular configuration of papaverine, for example, has not much in common with that of adenine. The inhibition of adenine transport by papaverine might be used to clarify the relation between transport and adenine phosphoribosyltransferase activity in platelets.

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