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TRANSPORT AND METABOLISM OF ADENOSINE IN HUMAN BLOOD PLATELETS

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SUMMARY

The uptake and metabolism of [^{14}C]- or [^{3}H]adenosine have been studied in suspensions of washed platelets and in platelet rich plasma. The appearance of radioactivity in the platelets and the formation of radioactive adenosine metabolites have been used to determine the uptake. Adenosine is transported into human blood platelets by two different systems: a low K_m system (9.8 μM) which is competitively inhibited by papaverine, and a high K_m system (9.4 mM) which is competitively inhibited by adenine. Adenosine transported via the low K_m system is probably directly incorporated into adenine nucleotides, while adenosine transported through the high K_m system arrives unchanged inside the platelet and is then converted into inosine and hypoxanthine or incorporated into adenine nucleotides.

INTRODUCTION

Several platelet functions, adenosine diphosphate- (ADP) induced aggregation in particular are inhibited by adenosine. This inhibition has been explained by: 1. Competition between adenosine and ADP receptors [1]. 2. Competition for a membrane adenosine triphosphate (ATP) which is needed for ADP-induced aggregation and also for adenosine transport [2]. 3. Stimulation of cyclic adenosine monophosphate (cyclic AMP) formation by adenosine [3]. An increase in intracellular cyclic AMP levels is accompanied by inhibition of platelet aggregation [4].

The first explanation is not likely, because adenosine monophosphate (AMP), which is structurally more related to ADP than adenosine, is not or is only a weak inhibitor [2, 5]. The second hypothesis was questioned when it was shown that papaverine, which inhibits adenosine uptake [6], simultaneously potentiated the inhibition of ADP induced aggregation by adenosine [7]. It should be pointed out, though, that papaverine is a potent inhibitor of cyclic AMP-phosphodiesterase [3] thus elevating the cyclic AMP levels (see hypothesis 3).

The inhibition at a certain level of cyclic AMP is more effective when adenosine

is used than when prostaglandin E_1, which is a more powerful stimulator of the cyclic AMP producing enzyme adenylcyclase, is used [3].

The precise mechanism of the inhibition of ADP induced aggregation by adenosine has thus not yet been clarified entirely. Detailed information on the adenosine uptake and metabolism in human platelets is lacking although this process has been studied more intensively in other cells [8–11]. The present paper reports on the kinetics of transport of adenosine and its metabolism in human blood platelets.

MATERIALS AND METHODS

[2,8-^{3}H]Adenosine spec. act. 30 Ci/mmol was purchased from New England Nuclear Corp., Boston; [U-^{14}C]adenosine spec. act. 500 Ci/mol was obtained from the Radiochemical Centre, Amersham. High voltage paper electrophoresis [12] of both radiochemicals showed that at least 92 and 95 %, respectively, of the radioactivity was adenosine. The adenosine was therefore used without further purification. The adenosine was diluted with non-radioactive adenosine before use. Adenosine, inosine, adenine, papaverine, 2-deoxy-D-glucose, *p*-hydroxymercuribenzoate, iodoacetamide, 2,4-dinitrofluorobenzene, *N*-acetyl imidazole and ouabain were obtained from Sigma Chemical Co., St. Louis; ATP, ADP, inosine monophosphate, AMP, hypoxanthine and β-mercaptoethanol were purchased from Boehringer, Mannheim; phlorizin was from Fluka, Switzerland.

Blood from donors, who had used no drugs, was collected into ethylenediaminotetra acetic acid, disodium salt (EDTA) (1 volume 0.027 M EDTA in 0.12 M NaCl pH 7.4 to 9 volumes blood) or into citrate (1 volume 0.11 M disodium citrate to 9 volumes blood) by venapuncture. After centrifugation (275 $\times g$ for 10 min at room temperature) the EDTA supernatant platelet rich plasma was washed twice with a buffer containing 0.103 M NaCl, 0.04 M NaH_2PO_4, 0.0047 M KH_2PO_4, 0.005 M glucose and 0.005 M EDTA, adjusted to pH 7.6 with 0.04 M NaOH according to Gaintner et al. [13]. 5 mg ml^{-1} bovine albumin was added to prevent enzyme leakage from the platelet [14]. This buffer is referred to as Gaintner-albumin buffer. Centrifugation during the washing procedure was carried out at 1000 $\times g$ for 10 min at 4 °C. No adenosine deaminase or adenosine kinase activity was observed in the supernatant of the final suspension of platelets, washed as described above, after centrifugation: 1000 $\times g$ at room temperature for 10 min.

Citrated platelet rich plasma was used in experiments in which platelets were separated from plasma by gelfiltration on a Sepharose 2 B (Pharmacia) column according to Tangen [15]. Platelet numbers were determined with the aid of a Coulter Counter.

Adenosine uptake was measured mainly by means of two different techniques.

(1) *High voltage electrophoresis.* 1.0 ml of the washed platelet suspension was preincubated for 5 min at 37 °C. 0.25 ml of a radioactive adenosine solution was added and samples of 0.25 ml were transferred to an Eppendorf tube in an ice bath, after 30 s and 2, 3 and 4 min of incubation. The Eppendorf tube contained 0.5 ml of a cold mixture of 1 volume 0.1 M EDTA and 9 volumes ethanol [16]. After thorough mixing the resulting mixtures were kept for at least 10 min at 0 °C, after which the tube was centrifuged (9500 $\times g$ for 2 min at room temperature) in an Eppendorf 3200 microcentrifuge. A sample of 50 μl of the supernatant was used for high voltage electropho-

resis at 60 V cm^{-1} for 1 h using a citrate buffer (pH 3.8) [17]. A solution containing eight purine compounds, ATP, ADP, IMP, AMP, hypoxanthine (Hyp), inosine, adenosine and adenine was used as markers. The different spots were visualized in ultraviolet light (259 nm), cut out and counted in a Packard model 3380 or model 2425 liquid scintillation spectrometer with a scintillation fluid consisting of toluene, 2,5-diphenyloxazole (PPO, 8 g · l^{-1}) and 2,2-'p-phenylen-bis-5-phenyloxazole (POPOP, 50 mg · l^{-1}).

A parallel electrophoresis of the radioactive adenosine was carried out for each experiment to check the purity. The adenosine uptake was calculated from the increase in radioactivity of Hyp+inosine and the adenine nucleotides. Inosine and hypoxanthine are not separated by the high voltage electrophoresis. In a series of experiments the Hyp+inosine spot was cut out, eluted with distilled water, concentrated in a nitrogen stream and solved in 0.1 ml water and subjected to electrophoresis in a 0.05 M borate buffer (pH 9.0) for 1 h at 60 V cm^{-1} [8]. In this buffer system hypoxanthine was separated from inosine.

(2) *Pellet technique.* 1.0 ml of a platelet suspension was preincubated for 5 min at 37 °C. 0.25 ml of a radioactive adenosine solution was then added and samples of 0.25 ml were transferred to individual Eppendorf tubes after 30 s and 2, 3 and 4 min of incubation. The tubes contained 0.25 ml ice cold Gaintner-albumin buffer, and the mixtures were centrifuged immediately in an Eppendorf 3200 microcentrifuge (9500 × *g* for 30 s at room temperature). The supernatant was discarded and the tube interior was rinsed twice with ice cold Gaintner-albumin buffer. The tube wall was then wiped with a cottonwool tipped stick and the pellet was solubilized in 0.25 ml 0.5 % Triton X-100 in saline by freezing and thawing twice. 0.1 ml portions from this mixture were used for liquid scintillation counting. The scintillation fluid contained 100 g naphthalene and 5 g PPO per liter of dioxane. 50 µl of each incubation mixture was counted directly in order to determine the specific radioactivity of the particular adenosine solution used.

The adenosine uptake in platelet rich plasma was measured by a rapid separation technique described by Karpatkin [18]. 0.75 ml of a mixture of 9.6 ml dibutylphtalate/0.4 ml Apiezon A was pipetted into each of four Eppendorf tubes. 0.45 ml platelet rich plasma was layered carefully on this mixture and after a preincubation for 5 min at 37 °C 0.05 ml radioactive adenosine solution was added to the tubes. After respectively 90, 60, 30 and 10 s incubation the tubes were centrifuged in an Eppendorf 3200 microcentrifuge (9500 × *g* for 30 s at room temperature). The supernatants were discarded and the tube walls were wiped twice with a cottonwool tipped stick. The pellet was solubilized in 0.5 % Triton X-100 in saline by freezing and thawing twice. From this suspension 0.1 ml was used for the determination of the radioactivity as outlined above. The adenosine uptake was expressed as pmol/min/10^9 platelets and calculated as initial rate from the linear part of the time course by taking points during the first 4 min (see results time course).

RESULTS

Time sequence and validity of the high voltage electrophoresis and pellet technique

The study of adenosine uptake was hampered by the observation that radioactive hypoxanthine and/or inosine and a small amount of adenine nucleotides

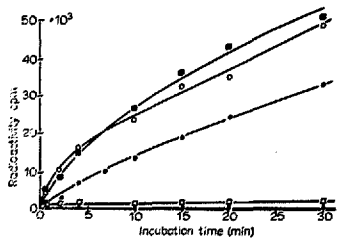

Fig. 1. Metabolism of adenosine at 60 μM. The pellet as well as the supernatant of a suspension of washed platelets $(9.1 \cdot 10^8\ ml)^{-1}$ incubated according to the pellet technique, were subjected to EDTA ethanol extraction and high voltage electrophoresis. ○–○, adenine nucleotides in pellet; □–□, hypoxanthine and inosine in pellet; △–△, adenosine in pellet; ●–●, adenine nucleotides in supernatant; ■–■, hypoxanthine and inosine in supernatant.

appeared in the supernatant after fast separation of the platelets from the medium by centrifugation at $9500 \times g$ (Fig. 1).

These metabolites were derived from intracellular conversion because no significant adenosine deaminase or adenosine kinase activity was observed in the supernatant (see Methods). Neglecting these metabolites would therefore lead to underestimation of the actual transport velocities. No free adenosine accumulated in the platelet pellet in the micromolar range. It was therefore possible to measure the transport velocity by carrying out high voltage electrophoresis of ethanol/EDTA extracts of the platelet suspension at the different time points after the addition of radioactive adenosine. The different radioactive metabolites were added and converted into pmol by comparison with a known amount of adenosine. Initial velocities were computed by drawing a line through time points obtained at 30 s and 2, 3 and 4 min incubation. This was justified by the linearity of the uptake during the first minutes (Fig. 2a). Fig. 2b shows that the uptake is lower when measured with the pellet technique. The differences in uptake measured by the high voltage electrophoresis and the pellet technique at 60 μM were in general not as large as in Fig. 2 (see also Fig. 8).

The situation at high adenosine concentrations is different. Free adenosine accumulates inside the platelet and the contribution of extracellular Hyp+inosine and adenine nucleotides was relatively unimportant (Figs. 3a and b). This made it possible to use the pellet technique in the millimolar range. The uptake was linear with time during the first 4 min (Fig. 4). The leakage of the metabolites from the platelets was taken into account in experiments in which more detailed information about the kinetic parameters was sought, by adding the metabolites found by high voltage electrophoresis of the supernatant. The difference was not very large however (Fig. 5).

Some attention was given to the question of whether a backflow of transported but not metabolized adenosine might lead to under estimation of the transport velocity and even to a pseudo saturation-curve for the concentration dependence at high adenosine levels. The latter possibility seems unlikely because of the linearity of the

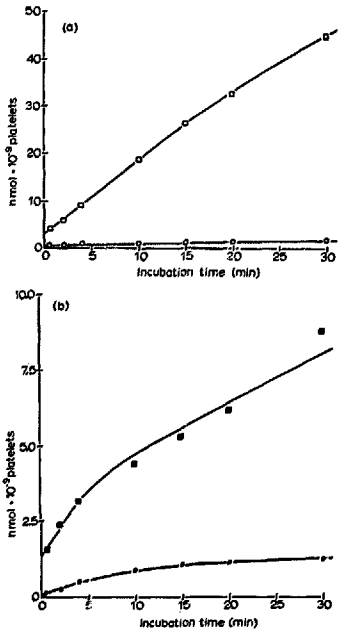

Fig. 2. Time dependence of adenosine uptake in the micromolar range. Comparison between the uptake of adenosine in a suspension of washed platelets when studied by (a) the high voltage electrophoresis technique (open symbols) and (b) the pellet technique (closed symbols) at 1.5 μM. (○–○ and ●–●) and at 60 μM (□–□ and ■–■).

uptake vs. time curves (Fig. 4). The problem was tackled in an experiment in which 5 mM radioactive adenosine was added to platelets. The radioactive platelet suspension was then diluted in 5 volumes of the buffer solution containing 5 mM "cold" adenosine. The accumulated radioactivity in the pellet showed no difference over the following 5 min and remained stable at the level already obtained after 3 min. (Results not shown.)

Concentration dependence of the adenosine uptake

a. Micromolar range. The rate of adenosine uptake seemed to be linearly related to the adenosine concentration above 30 μM, whereas, a steeper slope was

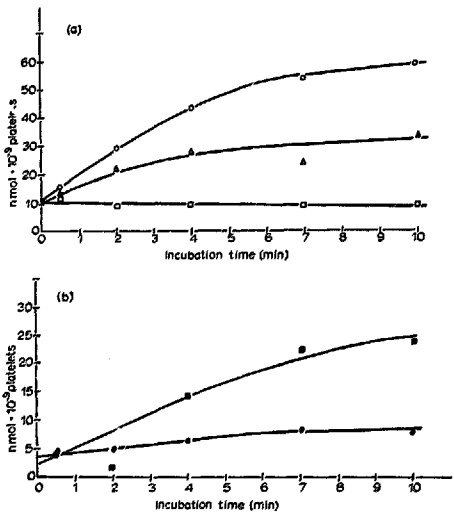

Fig. 3. Time dependence and metabolism of adenosine transport at 5 mM. For method used see Fig. 1. The amount of free adenosine in the pellet was corrected for trapped adenosine with the help of parallel experiments with [^{14}C]inulin. Platelets suspended in Gaintner-albumin ($1.6 \cdot 10^9$/ml). The uptake experiments do not go through the origin because of the time delay involved in rinsing the pellet. (a) □–□, hypoxanthine and inosine in the pellet; ○–○, adenine nucleotides in pellet; △–△, adenosine in pellet. (b) ■–■, hypoxanthine and inosine in supernatant; ●–●, adenine nucleotides in supernatant.

obtained at lower concentrations (Fig. 6). There appeared to be two components: a rectilinear and a curvilinear part. Subtraction of the rectilinear part was performed by drawing a line through the origin parallel to the observed rectilinear part of the uptake curve. A curvilinear part remained which conformed to a Michaelis-Menten curve. This allowed replotting of the remaining data in an Eadie Hofstee plot and a K_m value of 9.8±1.6 (S.E.) μM and a V of 797.6±79.5 (S.E.) pmol · min^{-1} · 10^{-9} platelets was calculated out of 6 experiments (Fig. 6). This transport system is further referred to as the low K_m system.

b. Millimolar range. The rectilinear part of the concentration dependence curve of the adenosine uptake was further studied by using adenosine concentrations in the millimolar range. A rectangular hyperbola was obtained which converted to a straight line in an Eadie Hofstee plot (Fig. 5). A K_m of 9.4±1.4 (S.E.) mM and a V of 106.5

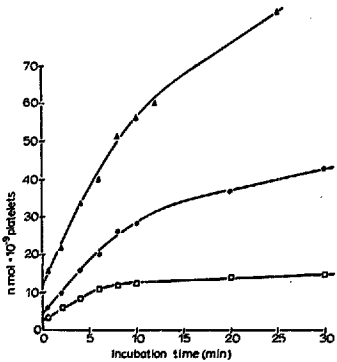

Fig. 4. Time dependence of adenosine uptake in the millimolar range. Adenosine uptake in the millimolar range as measured by the pellet technique: □-□, 1 mM; ●-●, 5 mM; △-△, 20 mM.

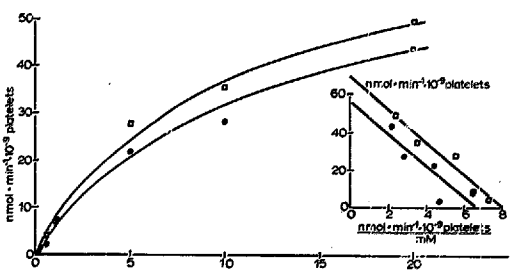

Fig. 5. Concentration dependence of adenosine uptake in the millimolar range. ●-●, pellet technique; □-□, pellet technique investigated with high voltage electrophoresis. A suspension of washed platelets in Gaintner-albumin was used. Inset, Eadie Hofstee plot of the curves. A typical experiment is shown.

±14.7 (S.E.) nmol · min^{-1} · 10^{-9} platelets were calculated. This transport system is further referred to as the high K_m system.

Inhibition by papaverine and adenine of the adenosine uptake

The low K_m system was almost completely inhibited by papaverine at a concen-

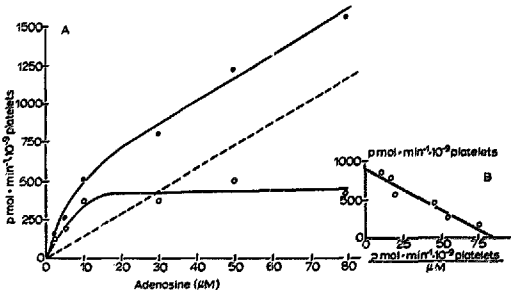

Fig. 6. Concentration dependence of adenosine uptake in the micromolar range. ●–●, total adenosine taken up, ○–○, adenosine transported after subtraction of the rectilinear part. Inset, Eadie Hofstee plot of the ○–○ curve. The high voltage electrophoresis technique was used. A typical experiment is given.

tration of 100 μM (Fig. 7). The effect of this concentration on the high K_m system was negligible. At 5 mM adenosine a slight inhibition was obtained with concentrations of 100 μM papaverine and above. The mode of inhibition of the low K_m system was further investigated. Papaverine acted as a competitive inhibitor of the low K_m system with a K_i of 26.6±4.0 (S.E.) μM ($n = 3$).

The high K_m system in the micromolar range was completely inhibited by 50 μM adenine (Fig. 7). The possibility that this was due to an inhibition of adenosine

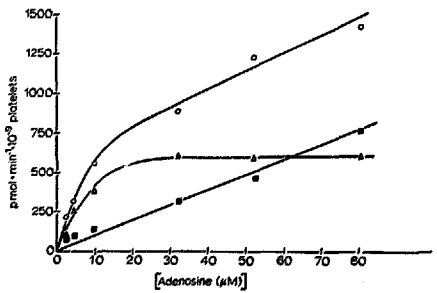

Fig. 7. Influence of adenine and papaverine on the uptake of adenosine in the micromolar range. ○–○, adenosine uptake in a suspension of washed platelets was measured by the high voltage electrophoresis technique. △–△, uptake in the presence of 50 μM adenine; ■–■, uptake in the presence of 100 μM papaverine (final concentration).

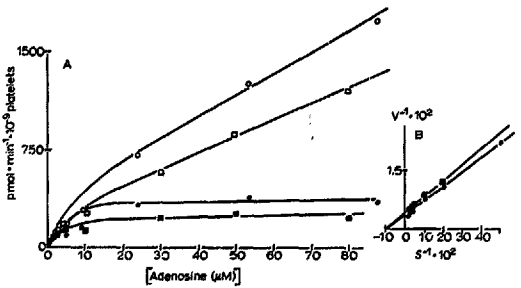

Fig. 8. Comparison of the uptake of adenosine in the micromolar range as measured with the high voltage electrophoresis technique and the pellet technique. ○–○, high voltage electrophoresis technique of suspension of washed platelets; □–□, pellet technique of same suspension; ●–●, high voltage electrophoresis technique after subtraction of rectilinear part; ■–■, pellet technique after subtraction of rectilinear part. Inset, Lineweaver Burk plot of the low K_m system. No differences in K_m and only some difference in V values were observed.

metabolism after transport was excluded in experiments in which adenine and labelled adenosine were added to platelet lysate. No inhibition of adenosine kinase or adenosine deaminase activity was observed (results not shown).

The character of the adenine inhibition of transport was investigated in the millimolar range by means of the pellet technique. This was justified because the differences found between the high voltage electrophoresis technique and the pellet technique for the high K_m system in the micromolar range were of much less importance in the millimolar range (Fig. 5). A competitive type of inhibition by adenine was observed with a K_i value of 5.8±2.9 (S.E.) μM in the three experiments performed (Fig. 7).

Inhibition by metabolic inhibitors

The glycolytic inhibitor 2-deoxy-D-glucose (7 mM and 10 min preincubation) and the oxidative phosphorylation inhibitor antimycin A (250 ng/ml, no preincubation) inhibited the uptake through the low K_m system (Fig. 9a) in a non competitive way. The high K_m system was inhibited in a similar way (Fig. 9b).

Intracellular metabolism of adenosine

(*a*) *Micromolar range.* The amount of adenosine incorporated into Hyp+inosine increased nearly linearly with the adenosine concentrations between 0 and 80 μM (Fig. 10a).

Hypoxanthine and inosine are not separated by the high voltage electrophoresis procedure. In a series of experiments (not shown) we detected that 80% of the Hyp+inosine spot was hypoxanthine. This distribution was found both in the micromolar and the millimolar range. The relative proportion of hypoxanthine increased slightly with time.

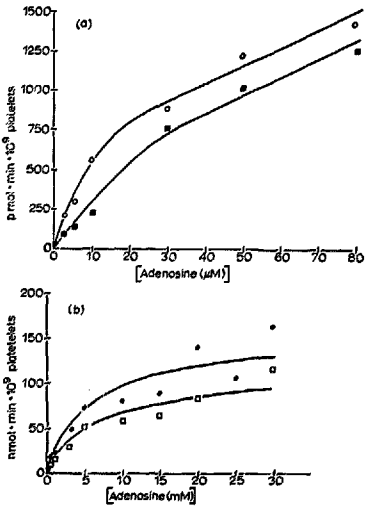

Fig. 9. Influence of 2 deoxy-D-glucose and Antimycin A. Washed platelets were suspended in Gaintner-albumin buffer and preincubated with 7 mM 2-deoxy-D-glucose for 10 min. Antimycin A was then added to a final concentration of 250 ng/ml and adenosine transport studied. (a) Uptake in the micromolar range, studied with high voltage electrophoresis technique. Open symbols give control values. (b) Uptake in the millimolar range studied with pellet technique, closed symbols give control.

The incorporation of adenosine into adenine nucleotides followed a saturable Michaelis-Menten like curve (Fig. 10b). Comparison of the curves obtained by subtraction of the high K_m system and the incorporation into adenine nucleotides showed that the incorporation into adenine nucleotides corresponded to the low K_m system, whereas the incorporation into Hyp+inosine was responsible for the linear part of the curve (Figs. 10a and b). Adenine had a negligible effect on the incorporation of adenosine into adenine nucleotides whereas, it inhibited strongly the appearance of Hyp+inosine as metabolites. Papaverine, 100 μM, strongly decreased the incorporation of adenosine in the adenine nucleotide but had also some effect on the incorporation in Hyp+inosine.

(*b*) *Millimolar range.* The fate of adenosine taken up at high adenosine concentrations could not be completely followed by the high voltage electrophoresis technique because a considerable part of adenosine taken up was present as free adenosine (Fig. 3a). The data obtained with high voltage electrophoresis of the pellet

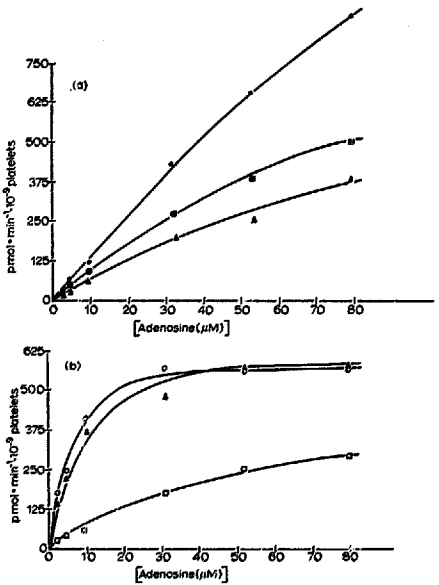

Fig. 10. Metabolism of adenosine in the micromolar range. Washed platelets suspended in Gaintner-albumin (a) depicts incorporation into hypoxanthine and inosine. (b) shows the incorporation into adenine nucleotides. ○–○ and ●–●, control; □–□ and ■–■, uptake in the presence of 100 μM papaverine; △–△ and ▲–▲, uptake in the presence of 50 μM adenine.

and supernatant showed that there was not only Hyp+inosine formation as with the high K_m system in the micromolar range but also a considerable adenine nucleotide formation at 5 mM (Fig. 3b).

Influence of pH, temperature, platelet concentration and suspending medium

Investigation of the various experimental conditions with the high voltage electrophoresis technique was not feasible because of the time-consuming nature of the technique. The pellet technique was therefore also used at low adenosine concentrations (1–100 μM). Separate experiments had shown that the K_m for both techniques was the same but that the V found for the pellet technique was lower than the V found for the high voltage electrophoresis technique (Fig. 8). At 5 μM which was chosen in many experiments as the "low" adenosine concentration the difference in uptake was negligible.

The influence of the pH was studied with the pellet technique. The platelets were resuspended after twice washing in isotonic Gaintner-albumin buffers of different pH values. Optima were found at pH 7.5 both at 5 μM (low K_m system) and at 5 mM (high K_m system). The curves had a symmetrical form.

Q_{10} values were obtained from Arrhenius plots at 5 μM and 5 mM with the pellet technique. The Q_{10} at 5 μM : 1.77±0.14 (S.E.M.), was significantly different from the Q_{10} at 5 mM : 1.31±0.05 (S.E.M.); (Student's t test $0.005 < P < 0.001$).

The rate of uptake varied linearly with the platelet number. Storage of a washed platelet suspension made no difference between 1 and 4 h after blood collection. Contaminating red blood cells (in general 0.5 %–1 % of the platelet number) only had influence when the percentage rose above 10 %.

No difference in uptake kinetics was observed between platelet rich plasma and washed platelets (studied with rapid separation according to Karpatkin [18]) and between gel filtered platelets and centrifuged platelets. The uptake in Tris-buffered saline with or without glucose added was the same as in Gaintner-albumin buffer. EDTA as an anticoagulant had no observable influence as may be concluded from the experiments with gel filtered platelets in which citrate was used as anti-coagulant.

Influence of different agents

A number of substances with an effect on cell metabolism and glucose transport were studied (Table I). No powerful effect of any of these substances was observed. Prostaglandin E_1 (10 μM), which has a definite inhibitory effect on adenine uptake [19] had no influence. Addition of ADP (25 μM) with stirring had no effect, again in contradiction with the uptake of adenine where ADP produces an increase in V and K_m [19].

TABLE I

INFLUENCE OF DIFFERENT METABOLIC INHIBITORS

Mean percentage of control ±S.E.M.

		5 μM	100 μM
Mercaptoethanol	1 mM	105.5±14.7	98.4± 0.2
p-Chloromercuribenzoate	10 μM	68.7±16.9	81.2± 2.6
Iodoacetamide	1 mM	73.0± 9.2	84.0± 9.9
2,4-dinitrofluorobenzene	240 μM	60.3± 8.1	109.0±20.9
Phlorizin	200 μM	59.6± 6.5	105.7±15.5
N-acetyl-imidazole	10 mM	96.3±17.1	79.4± 7.5
Ouabain	100 μM	86.5±10.3	70.1± 5.9

DISCUSSION

The mechanism of the adenosine transport between 0 and 80 μM adenosine is similar to that described for red cell ghosts [9], rabbit polymorphonuclear leukocytes [10] and guinea pig red blood cells [11]. The mechanism of uptake in these cells was considered to be a mixture of facilitated diffusion responsible for the initial

curvilinear part and simple diffusion responsible for the rectilinear part at higher adenosine concentration. Our results are not in agreement with the hypothesis that the rectilinear part is due to simple diffusion. We studied adenosine transport also at concentrations that were much higher than those utilized in the fore mentioned investigations. In this "millimolar range" we found a saturable Michaelis-Menten like curve suggesting a carrier mediated transport mechanism.

The K_m and V value observed are probably rather rough estimates because we were working at the solubility limit for adenosine (approx. $8 \text{ g} \cdot \text{l}^{-1}$).

A strong argument in favour of a carrier-mediated high K_m transport system is the powerful competitive inhibition of the uptake by adenine with a K_i value of 5.8 μM which suggests a very high affinity for the receptor. An alternative explanation for the adenine inhibition of adenosine uptake at high adenosine concentrations should be considered, the possibility that adenine inhibits directly the intracellular metabolism of adenosine. The decreased trapping of adenosine, either by adenosine kinase or adenosine deaminase might then lead to a stronger leaking of transported adenosine from the platelet. This seemed unlikely in view of the observations on adenosine transport and backflow in the millimolar range (see Results). A direct influence of adenine on intracellular metabolism was moreover excluded in an experiment in which we added adenine (200 μM) together with 2 mM [^{14}C]adenosine to a platelet lysate. No influence on the formation of Hyp+inosine or adenine nucleotides was demonstrated (results not shown).

Because of the powerful inhibition by adenine one could wonder whether the high K_m system would not be identical to the adenine uptake system that we described previously [19]. There are several observations about this adenine transport system, however, that are in conflict with this idea: the K_m for adenine is 10-times lower than the K_i for adenine inhibition of adenosine uptake and the K_i for adenosine (100 μM, ref. 19) inhibition of adenine uptake was 100-times lower than the K_m that we found for the high K_m system. Moreover, suggestive evidence for the close relation between the transport of adenine and adenine phosphoribosyl transferase (EC 2.4.2.7) [19, 20] makes adenosine transport by this system less likely. That the adenosine high K_m uptake system is not the same as the adenine uptake system is also supported by several other observations. The Q_{10} value for the adenosine transport through the high K_m system (1, 31) is much lower than that previously observed for the adenine uptake system (2, 53, ref. 19). Furthermore, prostaglandin E_1 and ADP which influence the adenine uptake system, have no effect on the high K_m system for adenosine.

The adenosine transported by way of the high K_m system arrives unchanged inside the platelet. This appears from the presence of free adenosine inside the platelet at high extracellular adenosine concentrations (Fig. 3a). At a lower concentration (Fig. 1) nearly all adenosine is immediately converted to inosine and hypoxanthine through the action of adenosine deaminase (EC 3.5.4.4.) and purine nucleoside phosphorylase (EC 2.4.2.1.). Hardly any adenosine transported through the high K_m system is converted into adenine nucleotides at concentrations between 0 and 80 μM adenosine. This can be derived from the identity between the linear part of the concentration dependence curve and the amount of hypoxanthine+inosine formed. It is confirmed by the observation that adenine, a powerful inhibitor of the high K_m system has hardly any effect on the incorporation of adenosine into nucleotides (Fig. 10b). The discrepancy between the appearance of hypoxanthine and inosine at low and high

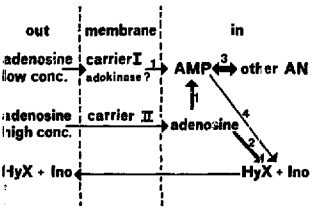

Fig. 11. Schematic diagram of adenosine transport and metabolism in human blood platelets. 1. Adenosine kinase; 2. Adenosine deaminase; 3. Adenylate kinase; 4. AMP deaminase and nucleoside phosphorylase. HyX, hypoxanthine; Ino, inosine; AN, adenine nucleotides.

adenosine concentrations is easily explained by the substrate inhibitory property of adenosine deaminase [8, 24]. This explains also why the pellet method can be used in the millimolar range for the high K_m system, whereas it reflects only parts of the low and high K_m system in the micromolar range (Fig. 8). The proposed relation of the two carrier mechanisms and the subsequent metabolism is summarized in Fig. 11.

The low K_m system is identical to the one described by Rozenberg and Holmsen [2]. It is of relatively major importance at low adenosine concentrations but nearly 20 % is already transported through the high K_m system at 5 μM adenosine. The adenosine transported through the low K_m system is protected in some way against degradation by adenosine deaminase. Several explanations may be offered for this phenomenon. It has been proposed that the relative K_m values of adenosine deaminase and adenosine kinase in the erythrocyte and the adenosine concentration determine whether the adenosine will be deaminated or phosphorylated [8]. This suggestion is not helpful for the explanation of adenosine metabolism in the human platelet. It does not explain why adenine blocks hypoxanthine formation concomitantly with transport through the high K_m system and why adenine nucleotide formation at low adenosine concentration is decreased when the low K_m system is inhibited by papaverine. Adenosine is degraded after transport in rat heart by adenosine deaminase [21], whereas, it is incorporated into adenine nucleotides in rabbit and cat heart [22, 23]. Rubio [25] postulated that adenosine kinase should be closely linked to the plasma membrane in the latter species. Some support for this hypothesis was found by de Jong and Kalkman [26]. They found that nearly all adenosine kinase was present in the cytosol of rat myocardium, whereas about 5 % was present in a 105 000 $\times g$ precipitate of other hearts (cat, rabbit and guinea pig). A simple spatial association of adenosine kinase with platelet membranes as observed by Holmsen et al. [27], is insufficient to explain why adenosine transported through the low K_m system is converted into adenine nucleotides only and adenosine transported through the high K_m system is preferentially degraded by adenosine deaminase. A close association of the low K_m carrier system with adenosine kinase should than be postulated. Phosphorylation may even be an integral part of the nucleoside transport as in bacterial transport involving group translocations [28]. A series of objections, mainly based on differences in kinetics and reaction to inhibitors between phosphorylation and transport,

have been raised against the idea of group translocation in mammals [29].

Moreover absence of transport in the presence of normal phosphorylating enzymes was used as an argument against group translocation. Many of these objections may be perhaps less strong than they appear. Other steps than phosphorylation may be rate limiting in a chain of processes, thus explaining the abnormal kinetic behaviour [30] and a membrane receptor, other than the phosphorylating enzyme may play an essential role. Indirect, but strong evidence in favour of the existence of group translocation in mammals was recently obtained by de Bruyn [31]. He discovered that erythrocytes of patients with the Lesch-Nyhan syndrome who are lacking hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8.) miss the low K_m transport system for hypoxanthine.

Our data are not in agreement with the observations of Haslam and Rosson [32]. These investigators found a maximal uptake at 10 μM adenosine which then decreased to a minimum of 45 % of this maximum at 100 μM and which then increased gradually at higher concentrations. The difference may be due to the technique used (double labelling experiments with [^{14}C]inuline a marker for trapping) which made it difficult to obtain exact data on the uptake rate at low concentrations.

Our results are also at variance with those of Jenkins et al. [33] who found little inhibition by adenine ($2.5 \cdot 10^{-4}$ M) and stronger inhibition by inosine ($2.5 \cdot 10^{-4}$ M). This may also be explained by technical differences. The authors used one concentration of adenosine and experimental conditions at which only the low K_m system would be assayed. We found no effect of adenine on the low K_m system and a relatively weak competitive inhibition by inosine (unpublished).

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