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INHIBITION OF ADENOSINE UPTAKE IN HUMAN ERYTHROCYTES BY ADENOSINE-5′-CARBOXAMIDES, XYLOSYLADENINE, DIPYRIDAMOLE, HEXOBENDINE, AND *p*-NITROBENZYLTHIOGUANOSINE

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Abstract—Adenosine uptake in human erythrocytes at 0° consists of a saturable and a concentration-proportional component, the latter seems to represent uptake into a pericellular compartment inaccessible to inulin. Xylosyladenine and derivatives of adenosine-5′-carboxamide were found to be weak inhibitors of the saturable component of adenosine uptake with apparent K_i values at least one order of magnitude higher than the apparent K_m for adenosine (2.4×10^{-6} M). The affinity of the adenine nucleosides to the saturable uptake process appears to depend not only on the 3′-hydroxyl group and its erythro-configuration but also on the 5′-substituent. Dipyridamole, hexobendine, and *p*-nitrobenzylthioguanosine, by contrast, had K_i values at least one order of magnitude lower than the K_m for adenosine. The steric requirements for binding of the adenine furanosides to the putative smooth muscle receptors mediating vasodilation, on the the one hand, and to the saturable cellular uptake mechanism, on the other hand, were found to be different.

The action of the potent coronary vasodilator adenosine is very short in duration due to rapid cellular uptake by erythrocytes and myocardial tissue [1–3] and conversion to nucleotides by adenosine kinase or to inosine by adenosine deaminase [4–6]. In order to prolong the action and to obtain compounds with vasodilating properties even after oral administration, amides of adenosine-5′-carboxylic acid were produced, because substitution in the 5′-position influences the enzymatic recognition by adenosine deaminase [7, 8] and prevents phosphorylation. Indeed, these compounds were shown to be considerably stronger and longer acting vasodilators than adenosine [9, 10]. This effect of the adenosine derivates may be brought about by three mechanisms: (1) by an intrinsic activity of the agents at the putative adenosine receptor on the vascular smooth muscle, (2) by inhibition of extracellular adenosine degradation, or (3) by inhibition of cellular uptake of endogenous adenosine.

It is the purpose of the present investigation to test this third possible mechanism of action by examining the influence of adenosine-5′-carboxamides on the cellular uptake of adenosine in comparison to the effects of dipyridamole, hexobendine, and *p*-nitrobenzylthioguanosine (NBTGR*), well known inhibitors of cellular nucleoside uptake [11–16]. The 3′-epimer of adenosine, xylosyladenine, was also included in the study, since this compound may provide additional information about the steric requirements of the nucleoside uptake process. Erythrocytes were used as a model system for this uptake study because (a) these readily available cells resemble myocardial cells in their ability to rapidly incorporate adenosine [6, 17] and (b) the concentration of substrate in the extracellular space can be followed more closely than in preparations such as perfused heart, since the cells may be quickly separated from the incubation medium.

Abbreviations—NBTGR—*p*-Nitrobenzylthioguanosine; ACM—adenosine-5′-carboxamide; AECM—adenosine-5′-(*N*-ethyl-carboxamide); B 744-97—3′,4′-dideoxy-adenosine-5′-(*N*-ethyl-carboxamide); B 744-98—2′,3′-*O*-methoxy-ethylidene-adenosine-5′-(*N*-ethyl-carboxamide); B 744-99—2′,3′-di-*O*-nitro-adenosine-5′-(*N*-ethyl-carboxamide).

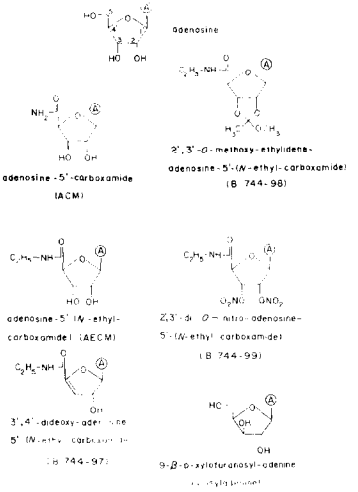
MATERIALS AND METHODS

Materials

[8-^{14}C]Adenosine (50 mCi/mmole) and [^{3}H]inulin (0.5 mCi/mg) were obtained from the Radiochemical Centre, Amersham, U.K. Unlabelled adenosine was purchased from Boehringer Mannheim, FRG. *p*-Nitrobenzylthioguanosine (NBTGR) was generously supplied by Dr. A. R. P. Paterson, University of Alberta, Cancer Research Unit, Edmonton, Alberta, Canada, hexobendine by Chemie-Linz, Austria, and dipyridamole by Bender & Co, Vienna, Austria. The adenine furanosides (Table 1) adenosine-5′-carboxamide (ACM), adenosine-5-(*N*-ethyl-carboxamide) (AECM), 3′,4′-dideoxy-adenosine-5′-(*N*-ethyl-carboxamide) (B 744-97; synthetized by Dr. Mengel, University of Konstanz, FRG), 2′,3′-*O*-methoxy-ethylidene-adenosine-5′-(*N*-ethyl-carboxamide)(B-744-98), 2′,3′-di-*O*-nitro-adenosine-5′-(*N*-ethyl-carboxamide) (B 744-99), and 9-β-D-xylofuranosyl-adenine (xylosyl-adenine) were kindly provided by Byk Gulden Lomberg, Chem. Fabr., Konstanz, FRG.

Experimental procedure. Blood was collected from healthy human volunteers with heparin as anticoagulant and used immediately for the experiments. Red blood cells were separated by centrifugation

Table 1. Structure of adenosine and the tested adenine (A) furanosides



at 760 *g* for 15 min followed by removal of the supernatant and buffy coat. Subsequently, the erythrocytes were resuspended in buffered (pH 7.4) saline of the following mM composition: NaCl 125, KCl 4.3, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, Tris-(hydroxymethyl)-aminomethan 27, HCl 23, glucose 5. This washing procedure, which was performed at 0°, was repeated five times.

Incubation was initiated by adding 0.5 ml erythrocyte suspension to 1 ml saline containing [^{14}C] adenosine (concentration range 0.2–1000 μM) with or without one of the tested inhibitors. In addition the incubation medium contained [^{3}H] inulin as an extracellular marker. The final haematocrit in the incubation system was 15 per cent.

At the end of the incubation period uptake was stopped by the addition of 2 μM (final concentration) NBTGR [15, 16]. Then the erythrocyte suspension was immediately centrifuged at 850 *g* for 5 min and the radioactivity determined in aliquots of the supernatant after addition of 9 ml of Bray's scintillator [18] in a Packard Scintillation Spectrometer, Tri Carb, Model 3380. 0.2 ml of the erythrocyte pellet were mixed with 0.5 ml 1 N perchloric acid, neutralized with K_2CO_3, and the radioactivity of the extract was measured. Counted radioactivity was corrected for quenching by use of internal standards.

A critical question for the measurement of transport rates is the rapidity with which adenosine uptake is terminated by NBTGR. This point was examined by testing whether the cellular ^{14}C-content increases after the addition of 2 μM NBTGR, a concentration more than one order of magnitude higher than the K_i of NBTGR for adenosine uptake inhibition, as pointed out later (Table 4). Table 2 contains data of experiments in which after a 10-sec incubation of the erythrocytes with either 0.3 or 300 μM [^{14}C] adenosine 2 μM NBTGR was added. After a further incubation period of the indicated time the cellular ^{14}C-content was determined in the usual manner. It is clear from Table 2 that with both a low and a high [^{14}C] adenosine concentration the amount of label taken up by the

Table 2. Uptake of adenosine (nmoles. 10 sec^{-1} ml^{-1} cell volume) in human erythrocytes in dependence on the time of incubation with 2 μM NBTGR which was added 10 sec after the initiation of the adenosine uptake

Adenosine conc. in the incubation medium	0.3 μM	300 μM
Time of incubation with 2 μM NBTGR		
7 Sec*	0.34 ± 0.02†	33.2 ± 9.8
20 Sec	0.32 ± 0.02	38.9 ± 4.8
1 Min	0.29 ± 0.04	30.9 ± 0.3
3 Min	0.30 ± 0.03	36.0 ± 2.9
10 Min	0.30 ± 0.04	32.9 ± 2.2

*The shortest time of incubation with NBTGR was approximately 7 sec, the time necessary for the centrifuge to reach 850 *g*.

†Results are expressed as means ± S.E.M.

cells does not increase with prolonged incubation in the presence of 2 μM NBTGR. It is therefore concluded that NBTGR very rapidly terminates adenosine uptake at 0°. The practically instantaneous onset of nucleoside transport inhibition in human erythrocytes was also demonstrated for the NBTGR-analogue 2-hydroxy-5-nitrobenzylthioguanosine [19, 20].

Neutralized acid extracts of the supernatants were subjected to thin layer chromatography on silica-gel plates (Kieselgel 60 F 254, E. Merck, Darmstadt, FGR) using *n*-butanol–acetone–NH_3 (33%)–H_2O = 50:40:3:14 (by volume) as the solvent system [21]. The radioactivity of the various chromatogram sections was determined directly by liquid scintillation counting after identification by use of appropriate carrier nucleobases and nucleosides which were visualized by fluorescence quenching.

Calculations. Uptake of [^{14}C] adenosine (nmoles. 10 sec^{-1}.ml^{-1} cell volume) was calculated from the difference between total ^{14}C-activity in the erythrocyte pellet and that in the extracellular space (inulin space).

The parameters for adenosine uptake and its inhibition were calculated by least square approximations of parameter estimates to the experimental values [22, 23] employing the general equation for linear mixed inhibition [24] plus a concentration-proportional term [23]:

$$v = \frac{VS}{K_m(1 + I/K_i) + S(1 + I/K_i')} + PS, \qquad (1)$$

where v is the total uptake/10 sec, S the substrate concentration, I the inhibitor concentration, K_m the half-saturation constant, and V the maximum rate of uptake by the saturable component of uptake; K_i and K_i' represent the dissociation constants of the carrier-inhibitor complex and the carrier–inhibitor–substrate complex, respectively, where the term carrier does not imply a certain molecular mechanism of transport, but merely stands for the saturable component of the uptake process; P is the proportionality coefficient for a concentration–proportional component of uptake. Weighting of the experimental data for the approximation procedure was performed

according to Ottaway [25] by dividing the deviation of each data point by the mean of the observed value and the value calculated by use of the parameter estimates. All calculations were carried out on a pdp 11/10 computer of Digital Equipment Corp., Maynard, Mass., U.S.A.

RESULTS

Preliminary experiments had shown that adenosine uptake in human erythrocytes at 37° proceeds too rapidly to resolve the initial uptake velocity. Therefore [^{14}C] adenosine uptake was studied at 0°. Figure 1 illustrates the time-course of the ^{14}C-incorporation, expressed as normalized units (cellular ^{14}C-concentration divided by the ^{14}C-concentration in the incubation medium). With 0.5 μM [^{14}C]-adenosine the cellular ^{14}C-accumulation appeared to increase linearly with time up to 10 sec, but later the uptake velocity decreased. With 200 μM [^{14}C]-adenosine, on the other hand, the increase in cellular ^{14}C-accumulation seemed to proceed linearly for even longer incubation periods than 10 sec.

However, in the presence of 2 μM NBTGR the cellular ^{14}C-content was independent of the duration of incubation and proportional to the adenosine concentration in the incubation medium (~0.1 normalized units). Further, the same value for the amount of [^{14}C] associated with the cells was derived when the time-course of the cellular incorporation in the absence of NBTGR was extrapolated to time $t = 0$ (Fig. 1). These findings suggest the existence of an inulin-inaccessible space in the immediate vicinity of the erythrocytes which is independent of the presence of NBTGR. The amount of [^{14}C] taken up into this compartment is part of the concentration proportional term of equation (1).

*Incubation medium, from which the erythrocytes were separated by centrifugation, did not degrade adenosine.

Table 3. Distribution of ^{14}C-activity in the incubation medium (in per cent of total ^{14}C-activity) after 10 sec of incubation with 0.6 μM (^{14}C] adenosine in the absence (control) and presence of 0.4μM NBTGR in comparison to the corresponding distribution in the stock solution

	Stock	0.6 μM Adenosine Control	0.6 μM Adenosine +0.4 μM NBTGR
Adenosine	95.5 ± 3.0*	85.2 ± 1.3	85.3 ± 2.8
Inosine	0.4 ± 0.2	8.1 ± 1.0	9.7 ± 0.9
Hypoxanthine	1.8 ± 1.3	5.1 ± 0.4	2.9 ± 1.5
Adenine	0.7 ± 0.1	0.5 ± 0.1	0.9 ± 0.6

*Results are expressed as means ± S.E.M.

If uptake is unidirectional up to 10 sec of incubation, as suggested by the linearity in the rise in cellular ^{14}C-content, no backflux of adenosine metabolites should occur within this time period. The results of the radiochromatographic analysis of the ^{14}C-activity in the suspension medium after 10 sec of incubation with 0.6 μM [^{14}C]adenosine are given in Table 3. Within this incubation period about 10 per cent of the adenosine originally added were converted to inosine and hypoxanthine. This finding seems to indicate that labelled adenosine-metabolites were released from the erythrocytes, since the incubation medium itself did not contain detectable adenosine deaminase activity.* However, in the presence of 0.4 μM NBTGR the same fraction of adenosine was converted to inosine and hypoxanthine as in the absence of NBTGR, although the cellular uptake, corrected for the inulin-inaccessible space, was reduced by 92 per cent. This finding suggests that adenosine may be deaminated at the outer surface of the cell membrane. Indeed, a relatively high specific activity of adenosine deaminase has been shown to be associated with the erythrocyte membrane

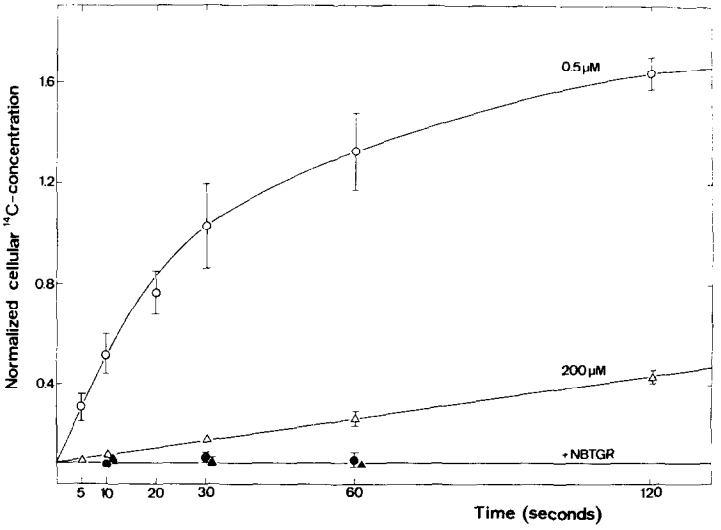


Fig. 1. Time-dependence of the cellular ^{14}C-uptake, expresses as cellular ^{14}C-concentration divided by the ^{14}C-concentration in the incubation medium. Human erythrocytes were incubated at 0° with 0.5 μM (circles) or 200 μM (triangles) [^{14}C]adenosine in the absence (open symbols) or presence (closed symbols) of 2 μM NBTGR. Each point represents the mean ± S.E.M. of three to eight experiments. Note (a) the time-independence of the cellular uptake in the presence of NBTGR, and (b) the common intercept of all time-courses on the ordinate at 0.1 normalized units.

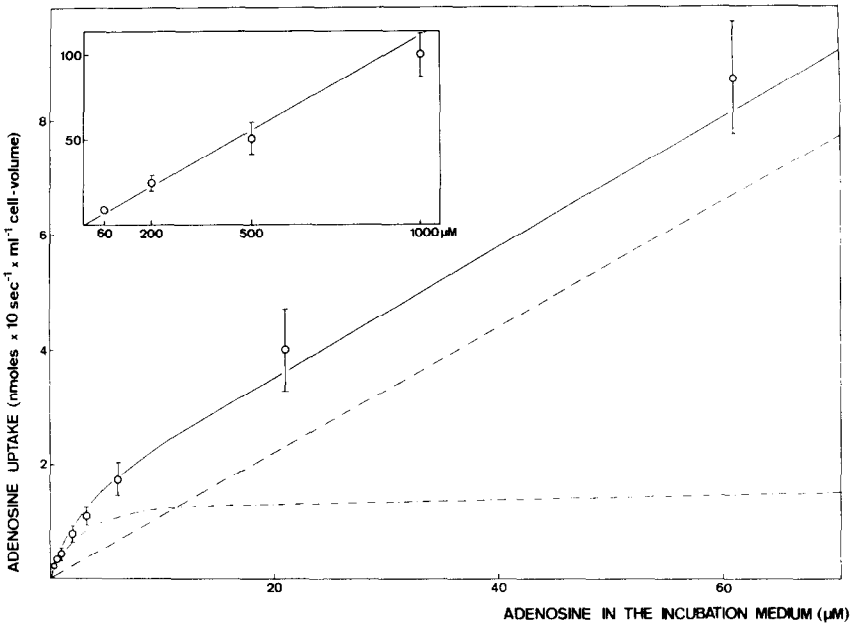


Fig. 2. Adenosine uptake in human erythrocytes at 0° as a function of the adenosine concentration in the incubation medium. Each point represents the mean ± S.D. of 12 experiments. The unbroken curve was calculated by a least square approximation of the parameters of the equation $v = (VS/K_m + S) + PS$ [23]: the dashed curve gives the concentration-proportional component of uptake, the dotted curve the saturable component. The inset depicts the adenosine uptake at substrate concentrations >60 μM.

[17]. Evidence similar to that of the present study was reported by Pfleger *et al.* [26] for washed guinea pig erythrocytes: although 1 μM dipyridamole completely blocked adenosine uptake, the rate of adenosine degradation was not inhibited. These authors therefore concluded that adenosine degradation is, at least in part, not connected to adenosine entry into the erythrocytes.

Since (a) the adenosine uptake, above that fraction which enters the inulin-inaccessible space, was linear up to 10 sec at 0°, and (b) the emergence of adenosine degradation products in the incubation medium was not a function of the rate of cellular adenosine uptake, the assumption seems reasonable that the determined 10-sec uptake values represent good estimates for the unidirectional influx of adenosine at 0°. Therefore this incubation period was chosen for all further experiments. The rate of adenosine uptake into human erythrocytes as a function of the adenosine concentration in the incubation medium is illustrated in Fig. 2. It is apparent that adenosine uptake may be divided into two components, one of which exhibited saturation kinetics, whereas the other was proportional to the adenosine concentration in the incubation medium. The apparent K_m (K_m^{app}) of the saturable uptake process was calculated to be 2.38 ± 0.15 μM with an apparent maximum rate of uptake (V^{app}) of 1.46 ± 0.10 nmoles. 10 $sec^{-1}.ml^{-1}$ cell volume. P, the proportionality coefficient for the non-saturable component, was found to be 0.10 ±0.01 per 10 sec.

Figure 3 gives examples of the influence of the compounds tested on saturable adenosine uptake, using the graphic method of the direct linear plot [27], which obeys the Michaelis–Menten equation and provides unbiased estimates of the kinetic constants. The type of inhibition elicited by the individual compounds varied: (1) xylosyladenine decreased the apparent affinity of the uptake system for adenosine without altering the apparent maximum rate of transport (competitive inhibition); (2) AECM also decreased the apparent affinity, but, in addition, decreased V^{app} (mixed type inhibition); (3) NBTGR reduced V^{app} without affecting the affinity (non-competitive inhibition); and (4) compound B 744-99 decreased the apparent affinity of the saturable uptake system for adenosine and, by contrast, increased the apparent maximum rate of incorporation in a concentration-dependent manner, suggestive of some kind of positive cooperativity.

Table 4 contains the combined data on the inhibition kinetics of adenosine uptake in erythrocytes, calculated by approximating parameter estimates of equation (1) to the experimental values. At least three different concentrations of each inhibitor were tested with several adenosine concentrations below and above K_m^{app}. P_I and P_C represent the proportionality coefficients for the non-saturable component of adenosine uptake in the presence of an inhibitor and in its absence (control), respectively. None of the agents tested significantly altered the concentration-proportional amount of ^{14}C in the erythrocyte fraction (Table 4).

NBTGR was the strongest inhibitor of saturable adenosine uptake with an inhibition constant more than one order of magnitude lower than the K_m^{app} for adenosine. Since K_i and K_i' were of similar magnitude with NBTGR (Table 4) the type of inhibition elicited

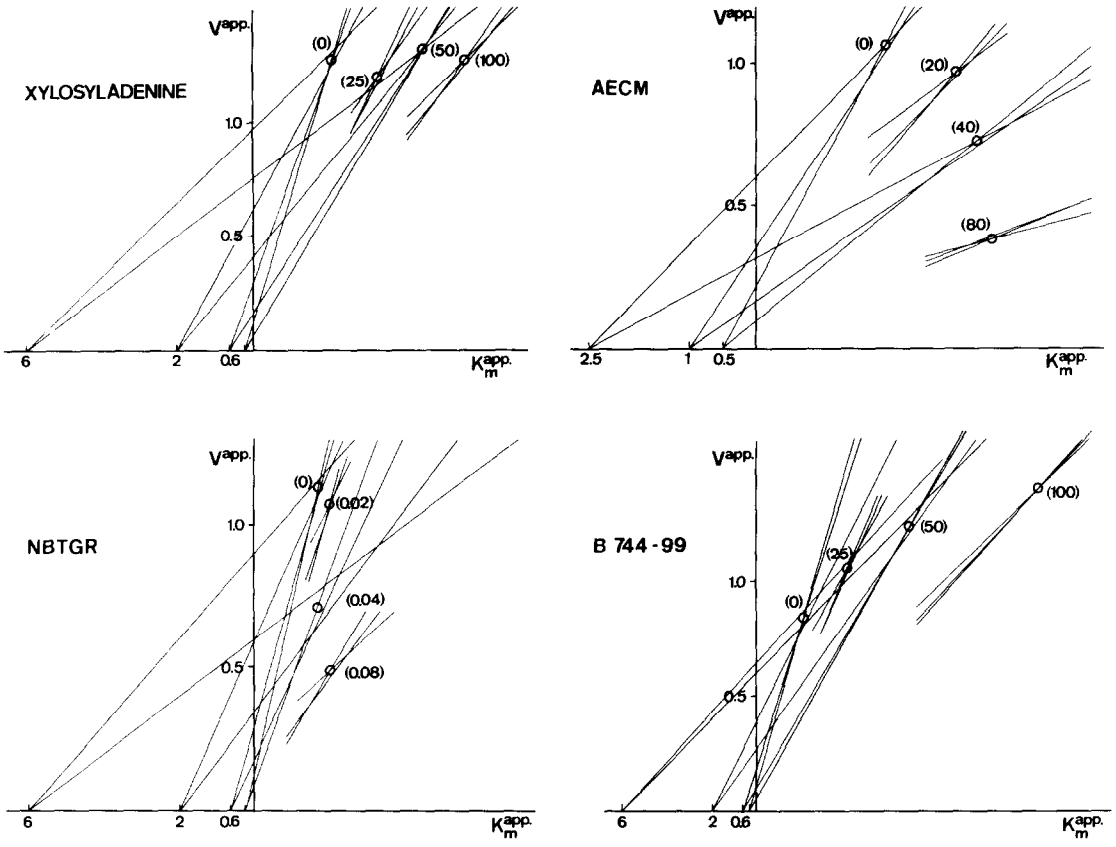


Fig. 3. Diagnosis of the inhibition type of saturable adenosine uptake (total uptake minus non-saturable component) in human erythrocytes caused by xylosyladenine, adenosine-5′-(*N*-ethyl-carboxamide) (AECM), *p*-nitrobenzylthioguanosine (NBTGR), and the 2′,3′-dinitro derivative of AECM (B 744-98) by use of direct linear plots according to Eisenthal and Cornish-Bowden [27]. For each set of intersections the median designating the estimate of the apparent K_m (μM) and V (nmoles. 10 sec^{-1} ml^{-1} cell volume) is shown as an empty circle. The concentrations of the individual inhibitors (μM) are given in parentheses next to the medians of the intersection points.

Table 4. Inhibition of adenosine uptake in human erythrocytes by dipyridamole, hexobendine, *p*-nitrobenzylthioguanosine (NBGTR) and the adenosine analogues given in Table 1*

	P_c ($10\,sec^{-1}$)	P_I	K_i (μM)	K_i' (μM)	*type of inhibition*
Dipyridamole	0.13 ± 0.01†	0.11 ± 0.01	0.23 ± 0.05	1.41 ± 0.80	mixed
Hexobendine	0.09 ± 0.01	0.08 ± 0.01	0.15 ± 0.02	13.90 ± 7.94	mixed
NBTGR	0.10 ± 0.01	0.11 ± 0.02	0.07 ± 0.04	0.08 ± 0.02	non-competitive
ACM	0.10 ± 0.04	0.09 ± 0.03	189.22 ± 15.54	> 1000	competitive
AECM	0.13 ± 0.01	0.12 ± 0.01	26.97 ± 2.65	129.38 ± 78.60	mixed
B 744-97	0.07 ± 0.01	0.07 ± 0.01	394.20 ± 121.70	−767.91 ± 150.30	mixed (‡)
B 744-98	0.13 ± 0.05	0.11 ± 0.03	54.22 ± 12.80	> 1000	competitive
B 744-99	0.10 ± 0.02	0.10 ± 0.02	56.06 ± 10.69	−258.17 ± 74.82	mixed (‡)
Xylosyladenine	0.11 ± 0.01	0.10 ± 0.01	52.22 ± 9.97	> 1000	competitive

*P_I and P_C, the coefficients for the concentration-proportional component of adenosine uptake in the presence of an inhibitor or in its absence (control), respectively, and K_i and K_i', the apparent dissociation constants of the carrier–inhibitor and the carrier–inhibitor–substrate complex, were calculated by approximation of the parameters of equation (1) to the experimental values. Inhibition was termed competitive when $K_i' > 1000\,\mu$M, non-competitive when $K_i' \approx K_i$, and mixed when K_i and K_i' were $< 1000\,\mu$M. Negative values for the calculated K_i' were obtained when V^{app} was increased (see Fig. 3), suggestive of positive cooperativity (‡).

†Results are expressed as the means ± S.E.M.

by this substance has to be classified as non-competitive [24]. Thus both the direct linear plot (Fig. 3) and the mathematical approximation procedure (Table 4) yielded the same type of inhibition. Agreement of the results obtained by the two independent methods was also found with the other agents tested. Dipyridamole and hexobendine were also potent inhibitors of saturable adenosine uptake, as shown previously [12-14, 28, 29]. The inhibition of nucleoside uptake by dipyridamole in dog erythrocytes [12], chick fibroblasts [30], and Novikoff rat hepatoma cells [31] was reported to be purely competitive on the basis of double reciprocal plots with only one concentration of dipyridamole, which seems insufficient to draw conclusions on the type of inhibition. In the present study, however, the inhibition of adenosine uptake by both dipyridamole and hexobendine was clearly of the mixed type (Table 4), implying that both the apparent affinity and the V^{app} of the saturable adenosine uptake were decreased.

In comparison with NBTGR, dipyridamole, and hexobendine the adenosine analogues tested were weak inhibitors of saturable adenosine uptake in human erythrocytes with K_i values one or two orders of magnitude higher that the K_m^{app} for adenosine. All of the adenosine analogues tested decreased the apparent affinity of the saturable uptake mechanism for adenosine. The inhibitory action of ACM, B 744-97, and xylosyladenine appeared to be of the purely competitive type, whereas AECM additionally decreased V^{app}. B 744-97 and B 744-99, on the other hand, not only decreased the apparent affinity of the saturable uptake mechanism for adenosine, but simultaneously increased V^{app}, as reflected by negative values for the calculated K_i' (Table 4).

DISCUSSION

In accordance with observations in human erythrocyte ghosts [17] and guinea pig erythrocytes [23] adenosine uptake in intact human red blood cells at 0° proceeds by both a saturable and non-saturable process within the range of adenosine concentrations studied (0.2–1000 μM). The saturable component of uptake indicates the existence of a specialized transport system that has been characterized as facilitated diffusion in a number of cell types [17, 33–35]. Although from the present results it is not possible to distinguish between the transport step and the subsequent metabolism in the overall uptake process, it has been repeatedly shown for the initial rates of uptake that (a) the cellular metabolic reactions of adenosine are distinct from transport and (b) the transport process represents the rate-limiting step in the incorporation of adenosine [31, 17, 32, 34]. It therefore seems justified to assume that the presented kinetic data represent primarily the transport mechanism.

The apparent affinity of adenosine to the saturable component of uptake in human erythrocytes (K_m^{app} = 2.38 μM) is well within the range of values reported for erythrocyte ghosts [17] and various other cell types of different species [3, 23, 29–32, 36].

The non-saturable component of adenosine uptake was characterized by a proportionality coefficient P of 0.1 per 10 sec, which was not altered by any of the compounds tested. This value agrees very well with the amount of ^{14}C attributed to the inulin-inaccessible space (see Fig. 1). It is therefore very probable that the concentration-proportional component of uptake is primarily brought about by uptake into the inulin-inaccessible space, whereas passive diffusion of adenosine across the cell membrane seems to be negligible. The observation that in the presence of NBTGR the content of this compartment did not change with time (Fig. 1), pH and temperature [37] also argues against the involvement of sizable transmembrane diffusion.*

From the different types of inhibition described in phenomenologic terms for the various adenosine analogues certain inferences concerning the mechanism of inhibition may be made, if interpreted in conventional terms [38]. The non-competitive type of inhibition found for NBTGR suggests that the inhibitor does not affect the binding of substrate to the uptake mechanism; this notion is consistent with the finding of Cass and Paterson [39] that the binding site of the nucleoside transport system for the NBTGR-analogue nitrobenzylthioinosine is distinct from the permeant site. The mixed-type inhibition, a mixture of competitive and non-competitive effects, found in the present study for the two long-acting coronary dilators dipyridamole and hexobendine may also be brought about by binding of these inhibitors to a site different from that for adenosine, which would not be surprising in view of the great discrepancy in chemical structure of the nucleoside and the inhibitors dipyridamole and hexobendine. An effect of these agents on membrane constituents other than the specific nucleoside binding site is also supported by the fact that dipyridamole has been found to inhibit the transfer of many substances of different chemical classes [34].

The adenosine analogues tested must be considered weak inhibitors of adenosine uptake since their K_i values far exceed the K_m^{app} for adenosine (Table 4). This finding is consistent with the observation of Cass and Paterson [40] that modifications of the sugar moiety are poorly tolerated by the uridine and thymidine transport system of human erythrocytes for which adenosine is also a substrate. Replacement of the 5′-carbinol group by 5′-*N*-ethyl-carboxamide (AECM) produced the strongest inhibitor amongst the adenosine analogues studied with a K_i of 27 μM, whereas adenosine-5′-carboxamide without the *N*-ethyl substitution (ACM) was a far weaker inhibitor with a K_i of 189 μM, suggesting that an apolar alkyl-substitution of the carboxamide group may facilitate interaction with the uptake mechanism. In addition, configuration and substitution at the 3′-position seems to be important, since the 3′-epimere of adenosine, xylosyladenine, had a K_i of 52 μM, 20 times the K_m^{app} of adenosine, and B 744-97, which lacks the hydroxyl group in this position, had a K_i of 400 μM. The 3′-position is also known as a

*Alternate explanations for the concentration-proportional component of adenosine uptake include adsorption and the existence of an additional fast, saturable transport process. However, both cases would imply a very high capacity of these mechanisms, saturation above 1000 μM, and lack of inhibition by the tested adenosine-analogues.

critical site for binding to the nucleoside transport system of other cells, since xylosyladenine was only a very weak inhibitor of nucleoside uptake in rabbit leucocytes [32] and did not influence uridine efflux in human erythrocytes [39] and adenosine uptake in canine heart [3].

No correlation was found between the *in vivo* coronary dilator effect of the adenosine analogues tested and their inhibitory action on adenosine uptake by erythrocytes: xylosyladenine, B 744-98, and B 744-99 inhibited adenosine uptake to a similar extent as reflected by their almost identical K_i values (Table 4); yet 744-98 possesses a very marked coronary dilator effect, the efficacy of B 744-99 is definitely smaller, whereas xylosyladenine is completely ineffective as a coronary dilator [35, 10]. This finding, together with the fact that the adenosine-5′-carboxamides are effective coronary dilators in concentrations far below their respective K_i values for adenosine uptake inhibiton excludes the possibility that the vasodilatory action of these compounds is brought about by inhibition of cellular adenosine uptake, if the myocardial adenosine uptake mechanism reacts in a manner similar to that of erythrocytes—an assumption which seems justified [13]. Additionally, the adenosine-5′-carboxamides appear to be very weak inhibitors of adenosine deaminase [9], (B. Plank and N. Kolassa, unpublished). Therefore, it may be concluded that these agents have an intrinsic activity at the smooth muscle receptor for adenosine, which mediates blood vessel relaxation, and furthermore that the steric requirements for binding of adenosine to the smooth muscle receptor and to the cellular uptake mechanism are different.

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REFERENCES

1. W. Kübler and H. J. Bretschneider, *Pflüger's Arch. ges. Physiol.* **277**, 141 (1963).
2. K. Pfleger, E. Seifen, and H. Schöndorf, *Biochem. Pharmac.* **18**, 43 (1969).
3. R. A. Olsson, M. K. Gentry, J. A. Snow, G. P. Frick, and R. S. Townsend, *Biochim. biophys. Acta* **311**, 242 (1973).
4. M. H. Maguire, M. C. Lucas, and J. F. Rettie, *Biochim. biophys. Acta* **262**, 108 (1972).
5. H. P. Baer, G. I. Drummond, and E. L. Duncan, *Molec. Pharmac.* **2**, 67 (1966).
6. M. H. Lerner and D. Rubinstein, *Biochim. biophys. Acta* **224**, 301 (1970).
7. A. Bloch, *J. med. Chem.* **10**, 908 (1967).
8. M. Ikehara and T. Fukui, *Biochim. biophys. Acta* **338**, 512 (1974)
9. H. H. Stein, P. Samoni, and R. M. Prasad, *Ann. N. Y. Acad. Sci.* **255**, 380 (1975).
10. G. Raberger, W. Schütz, and O. Kraupp, *Arch. int. Pharmacodyn. Ther* **230**, 140 (1977).
11. F. W. Koss, G. Beisenherz, and R. Maerkisch, *Arzneim. Forsch.* **12**, 1130 (1962).
12. W. Kübler and H. J. Bretschneider, *Pflüger's Arch. ges. Physiol.* **280**, 141 (1964).
13. K. Pfleger, I. Volkmer, N. Kolassa, and H. Stock, *Arzneim.-Forsch.* **19**, 1972 (1969).
14. N. Kolassa and K. Pfleger, *Biochem. Pharmac.* **24**, 154 (1975)
15. A. R. P. Paterson and J. M. Oliver, *Can. J. Biochem.* **49**, 271 (1971).
16. R. P. Agarwal and R. E. Parks, Jr., *Biochem. Pharmac.* **24**, 547 (1975).
17. J. Schrader, R. M. Berne, and R. Rubio, *Am. J. Physiol.* **223**, 159 (1972).
18. G. A. Bray, *Analyt. Biochem.* **1**, 279 (1960).
19. C. E. Cass and A. R. P. Paterson, *J. biol. Chem.* **247**, 3314 (1972).
20. D. B. Read, N. Bell, and R. N. McElhaney, *Analyt. Biochem.* **67**, 22 (1975).
21. N. Kolassa, H. Roos, and K. Pfleger, *J. Chromatogr.* **66**, 175 (1972).
22. J. Peil, *Biometr. Z.* **13**, 21 (1971).
23. H. Roos and K. Pfleger, *Molec. Pharmac.* **8**, 417 (1972).
24. A. Cornish-Bowden, *Biochem. J.* **137**, 143 (1974).
25. J. H. Ottaway, *Biochem. J.* **134**, 729 (1973).
26. K. Pfleger, D. Niederau, I. Volkmer, and H. Stock, *Naunyn-Schmiedeberg's Arch. Pharmac.* **265**, 118 (1969).
27. R. Eisenthal and A. Cornish-Bowden, *Biochem. J.* **139**, 715 (1974).
28. H. VanBelle, Europ. *J. Pharmac.* **11**, 241 (1970).
29. S. V. Hopkins and R. C. Goldie, *Biochem. Pharmac.* **20**, 3359 (1971).
30. C. Scholtissek, *Biochim. biophys. Acta* **158**, 435 (1968).
31. P. G. W. Plagemann, *Biochim. biophys. Acta* **233**, 688 (1971).
32. R. A. Taube and R. D. Berlin, *Biochim. biophys. Acta* **255**, 6 (1972).
33. P. G. W. Plagemann, *Archs Biochem. Biophys.* **140**, 223 (1970).
34. P. G. W. Plagemann and D. P. Richey, *Biochim. biophys. Acta* **344**, 263 (1974).
35. R. A. Olsson and R. E. Patterson, *Progr. Molec. Subcell. Biol.* **4**, 227 (1976).
36. N. Kolassa, R. Stengg, and K. Turnheim, *Can. J. Physiol. Pharmac.* **55**, 1033 (1977).
37. N. Kolassa, B. Plank, and K. Turnheim, *Eur. J. Pharmacol.* (in press).
38. M. Dixon and E. C. Webb, *Enzymes*, 2nd ed., Longman London (1971).
39. C. E. Cass and A. R. P. Paterson, *Biochim. biophys. Acta* **419**, 285 (1976).
40. C. E. and A. R. P. Paterson, *Biochim. biophys. Acta* **291**, 734 (1973).