Biochem. J. (1978) **170**, 265–271
Printed in Great Britain

Uptake and Metabolism of Adenosine by Pig Aortic Endothelial and Smooth-Muscle Cells in Culture

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(*Received* 4 *July* 1977)

1. Adenosine, a potent vasodilator, is transported very efficiently by pig aortic endothelium in monolayer culture (approx. 50pmol/min per 10^6 cells at 2μM). Uptake proceeds by diffusion at high (millimolar) substrate concentrations, and by two discrete transport processes (K_m approx. 3μM and 250μM) at lower concentrations. Over 90% of the adenosine taken up at 10μM or 100μM is rapidly converted into adenine nucleotides (mainly ATP). 2. The high-affinity process is selectively inhibited by dipyridamole and by nitrobenzylthioinosine. Adenine preferentially inhibits the lower-affinity process, papaverine inhibits both transport processes, and inosine has no significant effect. 3. Pig aortic smooth-muscle cells in culture show no high-affinity transport system for adenosine; uptake is much slower at low concentrations than that by endothelium (approx. 5pmol/min per 10^6 cells at 2μM). Over 80% of the incorporated adenosine at 10μM or 100μM is rapidly converted into adenine nucleotides. 4. The uptake of adenosine by smooth-muscle cells is powerfully inhibited by adenine, but dipyridamole is much less potent than in endothelium. 5. We conclude that endothelial cells are mainly responsible for the removal of circulating adenosine.

The removal of vasoactive agents from the bloodstream is an important factor in the maintenance of circulatory homoeostasis. There is considerable indirect evidence implicating the endothelial cell in the removal of certain vasoactive agents, such as 5-hydroxytryptamine (serotonin) (Strum & Junod, 1972), but a direct demonstration requires studies with isolated cells. With the advent of primary-cell-culture techniques such studies became feasible, and it has been shown that 5-hydroxytryptamine is transported and metabolized by isolated and cultured endothelial cells (Shepro *et al.*, 1975; Junod & Ody, 1977; Pearson *et al.*, 1977).

Adenosine is a potent vasodilator (Drury & Szent-Gyorgi, 1929), and, although it was previously believed that it was inactivated by deamination (Clarke *et al.*, 1952), more recent studies have provided indirect evidence suggesting that uptake into endothelium could be responsible for removing the circulating compound (Kolassa *et al.*, 1971).

We have now shown that pig aortic endothelial cells in culture take up adenosine very rapidly by two transport processes. In contrast, aortic smooth-muscle cells possess no high-affinity transport system for adenosine. From these experiments it appears that endothelial cells possess a capacity for adenosine transport that is more than sufficient to account for the observed removal of this compound *in vivo*.

Materials and Methods

Cell culture

Endothelial cells were as a routine established in culture from 1–10-day-old pigs of the Babraham herd (and occasionally from adult pigs) by collagenase treatment of thoracic aortas (Jaffe *et al.*, 1973) as described in detail by Pearson *et al.* (1977). Briefly, endothelial cells were obtained in small sheets after treatment of the aortic lumen (15min, 37°C) with 0.2% collagenase [type II; Aldrich Chemical Co., Gillingham, Dorset, U.K., or Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.] in Dulbecco's modification of Eagle's medium (Dulbecco & Freeman, 1959) containing penicillin (50 units/ml; 30μg/ml) + streptomycin (50μg/ml) (Crystamycin; Glaxo Laboratories, Greenford, Middx., U.K.) and kanamycin (100μg/ml) (Kanasyn; Winthrop Laboratories, Surbiton, Surrey, U.K.). The suspension was centrifuged (100*g* for 3min), and the cells from one aorta were resuspended in 5ml of growth medium + 20% (v/v) foetal bovine serum (Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.) and plated in a 25cm^2 Falcon tissue-culture flask [Becton Dickinson (U.K.) Ltd., Wembley, Middx., U.K.]. Growth medium was changed every 2 or 3 days, and the cells were subcultured (usually at 1:2 split ratio) when confluent by treatment with 0.1%

trypsin (1:250; Difco Laboratories, Detroit, MI, U.S.A.) + 0.025% EDTA in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (Dulbecco & Vogt, 1954). Cells were used for experiments after one to six subcultures.

Smooth-muscle cells were obtained in culture from explants of pig aortic media, as previously described for rabbit aortic smooth-muscle cells (Pearson, 1976).

Chemicals

[G-^{3}H]Adenosine (38 Ci/mmol), from The Radiochemical Centre, Amersham, Bucks, U.K., was diluted to 20 μM in 154 mM-NaCl and stored in 0.5 ml volumes at 4°C. Other reagents were obtained as below: adenosine (puriss.) from Koch–Light, Colnbrook, Bucks., U.K.; inosine from Sigma (London) Chemical Co.; papaverine sulphate from Macarthys Pharmaceuticals, Romford, Essex, U.K.; dipyridamole (Persantin injection) from Boehringer Ingelheim Ltd., Bracknell, Berks., U.K. Nitrobenzylthioinosine {6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine} was a generous gift from Dr. A. R. P. Paterson, University of Alberta Cancer Research Unit, Edmonton, Canada. All reagents were dissolved in 154 mM-NaCl immediately before use (except nitrobenzylthioinosine, in water) or diluted from small volumes of concentrated stock solutions stored at −20°C.

Adenosine uptake

Confluent monolayers of cells in 6 mm-diameter wells of Falcon Microtest II 96-well plates (approx. 10^4 cells/well) were used, after removal of growth medium and rinsing with serum-free medium. All incubations were at 37°C. Replicate pre-warmed incubation solutions (0.1 ml) were added simultaneously to each well from 1 ml disposable syringes [Becton–Dickinson (U.K.) Ltd.] held in place by a Perspex template over the Microtest plate. At the end of the incubation period the cells were rapidly rinsed three times with a large excess of ice-cold 154 mM-NaCl, left to drain, and then solubilized in 0.1 ml of 25 M-formic acid. The amount and distribution of incorporated radioactivity were not altered if the rinsing fluid contained an excess of unlabelled adenosine. Each formic acid digest was transferred, with one rinse of 0.1 ml of 154 mM-NaCl, to a scintillation vial containing 8 ml of scintillation fluid [1 litre of toluene + 5 g of 2,5-diphenyloxazole + 100 mg of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene + 500 ml of Emulsifier Mix no. 1 (Fisons Scientific Apparatus, Loughborough, Leics., U.K.)]. Radioactivity was determined in a Packard Tri-Carb liquid-scintillation spectrometer. Uptake in incubated samples was always corrected for non-specific binding or trapping of label, found in replicate samples in which the rinsing was performed immediately after addition of the incubation solution. The radioactivity associated with such samples was always less than 3 times background radiation, or less than 5% of the counts associated with incubated samples. Cell numbers were obtained from replicate wells by removing the cells with trypsin + EDTA, suspending them in 154 mM-NaCl and counting on a Coulter (Bedford, U.K.) model B electronic particle counter.

Measurement of adenosine metabolites

The products of [^{3}H]adenosine metabolism plus carrier ATP, ADP, AMP, IMP, adenosine, inosine and hypoxanthine were separated by t.l.c. on silica-gel-coated plastic sheets impregnated with fluorescent indicator (Polygram; Camlab, Cambridge, U.K.) by a slight modification of the method described by Norman *et al.* (1974). Briefly, after incubation with [^{3}H]adenosine and rinsing as described above, 50 μl of ice-cold 10% (w/v) trichloroacetic acid containing 20 p.p.m. of Bromocresol Green was added to each well. After 30 min on ice, 5 M-K_2CO_3 was added in sequential 1 μl volumes, with thorough mixing, until the indicator changed colour (about 4 μl of K_2CO_3). Samples (20 μl) were then chromatographed on 5 cm × 15 cm strips for 3 h at room temperature. R_F values were as follows: ATP 0.03, ADP 0.11, IMP 0.25, AMP 0.37, inosine 0.50, adenosine 0.61, hypoxanthine 0.61. Spots were located under u.v. light, outlined in pencil, cut out and transferred to scintillation vials. Distilled water (1 ml) and scintillation fluid (8 ml) were added to each vial and radioactivity was determined as above.

Results

Uptake by endothelial cells

Uptake of adenosine was linear with time for at least 30 min, at substrate concentrations of 2 μM, 100 μM and 1 mM (results not shown). There was no significant difference in the rates of uptake when measured in serum-free growth medium or in Ca^{2+}- and Mg^{2+}-free phosphate-buffered 154 mM-NaCl (Dulbecco & Vogt, 1954). Subsequent experiments were performed in phosphate-buffered 154 mM-NaCl by using incubation times of 20 min or less.

Uptake of adenosine was studied at concentrations between 0.5 μM and 10 mM. There appeared to be two discrete saturable transport systems, plus a non-saturable influx at high (millimolar) concentrations, assumed to be diffusion. The results of a representative experiment are shown in Fig. 1. A reciprocal plot (Lineweaver & Burk, 1934) from the data in Fig. 1(*a*) revealed a high-affinity transport site with apparent K_m of 3 μM and $V_{max.}$ approx. 90 pmol/min per 10^6 cells (Fig. 2*a*). This plot covers concentrations up to

10μM, where the contribution to the measured influx by the lower-affinity transport process and by diffusion was calculated to be below 5%. A similar plot was derived from the data in Fig. 1(*b*), with substrate concentrations of 0.1–2mM, by subtracting the contributions made by the saturated high-affinity site and by diffusion (130pmol/min per 10^6 cells at 1mM). This revealed a lower-affinity uptake site with apparent K_m approx. 250μM and $V_{max.}$ approx. 300pmol/min per 10^6 cells (Fig. 2*b*).

Uptake by smooth-muscle cells

As with endothelium, adenosine uptake by pig aortic smooth-muscle cells increased linearly with time of incubation for over 30min at 37°C. In contrast with endothelium, however, smooth-muscle cells showed no evidence of a saturable transport process at substrate concentrations of up to 1mM. Uptake was virtually linear with respect to substrate concentration over the range 2–20μM (Fig. 3*a*), and also from 0.05 to 1mM (Fig. 3*b*). This uptake represented about 5pmol/min per 10^6 cells at 2μM, only 10% of the uptake by endothelium at the same substrate concentration.

Metabolism

Distribution of radioactivity in the products of adenosine metabolism was determined after incu-

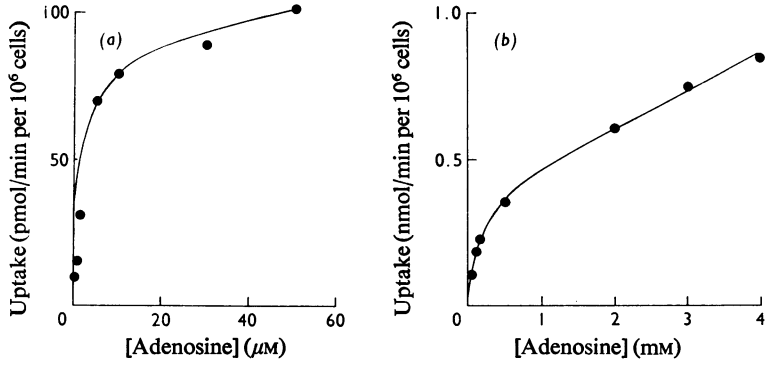

Fig. 1. *Concentration-dependence of adenosine uptake by cultured endothelial cells*
(*a*) 0–50μM-Adenosine, 15min incubations; (*b*) 50μM–4mM-adenosine, 20min incubations; as described in the text. Each point represents the mean of four observations, corrected for duplicate (non-incubated) controls at each adenosine concentration.

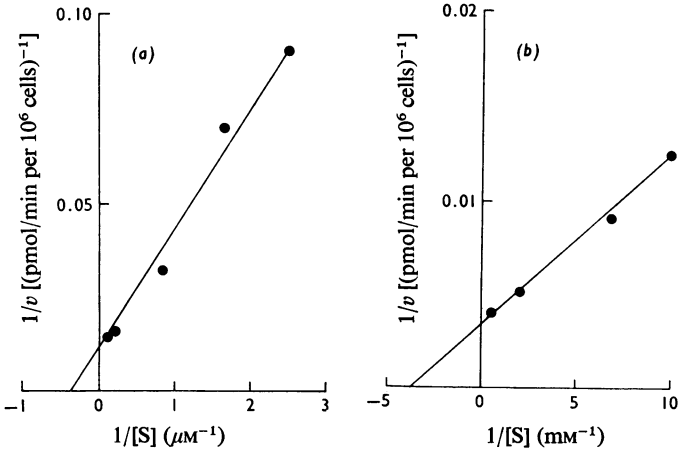

Fig. 2. *Determination of kinetic constants for adenosine uptake by cultured endothelial cells*
(*a*) 0.4–10μM-Adenosine, 15min incubations; (*b*) 0.1–2mM-adenosine, 20min incubations; as described in the text. Each point represents the mean of four observations, corrected for duplicate (non-incubated) controls at each adenosine concentration; those in (*b*) have also been corrected to remove the contributions from the non-saturable uptake component and the saturated high-affinity site.

bating cells with either 10 μM- or 100 μM-[^{3}H]adenosine for 20min at 37°C. At 10 μM-adenosine the endothelial-cell high-affinity transport process is saturated and represents over 80% of the total uptake. At 100 μM-adenosine the lower-affinity process is responsible for about 60% of the total uptake, whereas the non-saturable influx contributes less than 5% to the total uptake.

Over 92% of the radioactivity in endothelial cells was associated with adenine nucleotides at either substrate concentration (Table 1). The same pattern was observed with 5 μM- or 200 μM-adenosine, or with incubation periods as short as 5min (results not shown). The metabolism of [^{3}H]adenosine in smooth-muscle cells followed a broadly similar pattern, but with three points of difference: a slightly lower proportion of the radioactivity (approx. 85%) was associated with adenine nucleotides; there was a lower ratio of ATP/ADP; and a higher amount of ^{3}H was found in the adenosine + hypoxanthine fraction (Table 1).

Inhibition of uptake

We tested several potential inhibitors of uptake, with a view to discriminating pharmacologically between the transport processes. Among the agents tested were adenine, inosine, papaverine and dipyridamole. The last compound was of particular interest, because it has been shown to inhibit the uptake of adenosine by the lung, which contains the largest capillary endothelial network in the body (Kolassa *et al.*, 1971), and it was therefore tested more extensively. The effects of dipyridamole on adenosine uptake by endothelial cells, with substrate concentrations in the range 0.2–20 μM, are shown in Fig. 4. Even at 0.2 μM, dipyridamole greatly inhibited the uptake of adenosine. The adenosine influx remaining

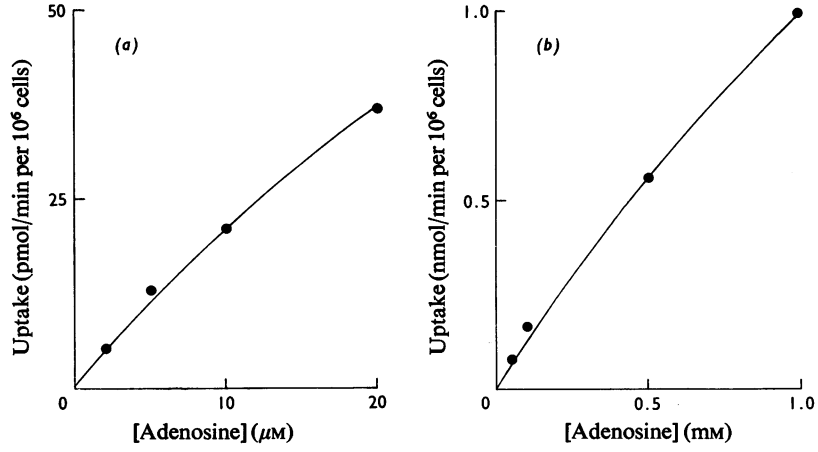

Fig. 3. *Concentration-dependence of adenosine uptake by cultured smooth-muscle cells*
(*a*) 2–20 μM-Adenosine; (*b*) 0.05–1 mM-adenosine; 15min incubations. Incubations were performed as described in the text. Each point represents the mean of four observations, corrected for duplicate (non-incubated) controls at each concentration.

Table 1. *Uptake and metabolism of adenosine by cultured endothelial and smooth-muscle cells*
Samples were incubated for 20min, as described in the text. Each value is the mean percentage of ^{3}H (±S.E.M., four observations) incorporated into each compound. Significant differences in the patterns of incorporation between 10 μM- and 100 μM-adenosine were found by *t* test: *0.001 < *P* < 0.01; †0.01 < *P* < 0.05.

	Endothelial cells		Smooth-muscle cells	
[Adenosine] in incubations ...	10 μM	100 μM	10 μM	100 μM
ATP	67.0±2.3	65.2±2.2	52.2±2.2†	43.0±2.6
ADP	17.9±0.7	18.8±0.8	28.1±2.5	34.3±2.3
IMP	2.5±0.1	2.0±0.0	1.7±0.1	2.6±0.3
AMP	9.8±0.8	8.9±1.1	6.2±0.4	5.6±0.4
Inosine	0.9±0.1	1.4±0.1	1.4±0.1	2.8±0.2
Adenosine + hypoxanthine	1.9±0.2*	3.7±0.2	10.5±1.7	11.3±2.8

in the presence of 0.2 μM-dipyridamole was apparently linear with respect to adenosine concentration (over the range used in this experiment), and the rate corresponded closely to that calculated for uptake by the lower-affinity process. Increasing the concentration of dipyridamole tenfold had little further effect on this residual adenosine influx, which suggested that dipyridamole was not a potent inhibitor of the lower-affinity transport process. We therefore tested several concentrations of dipyridamole (and of the other potential inhibitors) against adenosine uptake at substrate concentrations of 10 μM and 100 μM. The effects of dipyridamole, papaverine, adenine and inosine are shown in Table 2.

Dipyridamole inhibited the uptake of 10 μM-adenosine by over 60% at 0.1 μM, but had less effect against 100 μM-adenosine even at 100 μM. Nitrobenzylthioinosine was similar to dipyridamole in potency and in spectrum of activity (results not shown). Papaverine was approximately equally active against both substrate concentrations, inhibiting uptake by over 50% at 10 μM, whereas adenine was more effective against the higher substrate concentration: uptake of 100 μM-adenosine was 80% inhibited by 10 μM-adenine. Inosine had no significant effect on adenosine uptake.

The effects of dipyridamole, adenine, papaverine and inosine on adenosine uptake by smooth-muscle cells were also investigated (Table 3). Inosine again had little effect. Adenine was a powerful inhibitor (at least as potent as on endothelium), whereas papaverine was rather less effective on smooth muscle, and dipyridamole much less effective, compared with its effect on the uptake of 10 μM-adenosine by endothelium. In no instance was there a substantial difference in the potency of an inhibitor against substrate concentrations of 10 μM or 100 μM.

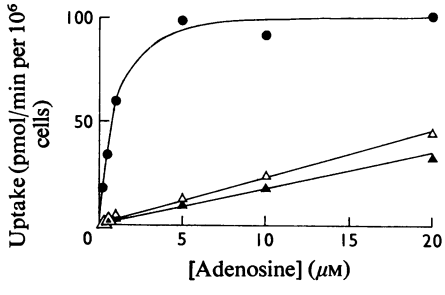

Fig. 4. *Inhibition by dipyridamole of adenosine uptake by cultured endothelial cells*

0.2–20 μM-Adenosine, 10 min incubations. ●, Adenosine alone; △, +0.2 μM-dipyridamole; ▲, +2 μM-dipyridamole. Incubations were performed as described in the text. The inhibitor was added simultaneously with the adenosine. Each point represents the mean of four observations, corrected for duplicate (non-incubated) controls.

Table 2. *Inhibition studies on the uptake of adenosine by cultured endothelial cells*

Samples were incubated for 20 min, as described in the text. Each inhibitor was added at the same time as adenosine. Results are given as percentage of control uptake (mean values ± S.E.M.; four observations for each drug, eight for each control).

[Adenosine] ...	10 μM	100 μM
Dipyridamole (μM)		
0	100±4	100±3
0.1	35±5	102±9
1	31±0.5	85±12
10	29±0.8	81±8
100	20±0.8	65±4
Papaverine (μM)		
0	100±3	100±2
1	88±2	70±7
10	41±3	40±2
100	21±1	10±2
Adenine (μM)		
0	100±5	100±3
1	70±8	55±2
10	43±3	20±1
100	41±4	9.5±0.3
Inosine (μM)		
0	100±5	100±3
1	97±6	101±7
10	87±8	92±5
100	84±6	100±1

Table 3. *Inhibition studies on the uptake of adenosine by cultured smooth-muscle cells*

Samples were incubated for 20 min, as described in the text. Each inhibitor was added at the same time as adenosine. Results are given as percentages of control uptake (mean values ± S.E.M.; four observations for each drug, eight for each control).

[Adenosine] ...	10 μM	100 μM
Control	100±13	100±4
Dipyridamole (μM)		
1	56±1	82±4
100	37±1	45±1
Papaverine (μM)		
10	58±3	81±4
100	25±1	36±1
Adenine (μM)		
1	52±2	74±3
10	26±4	31±3
100	14±2	30±2
Inosine (μM)		
10	87±10	84±6
100	94±14	81±4

Discussion

Adenosine is believed to be an endogenous regulator of coronary blood flow, and is released into the bloodstream after tissue damage, for example during myocardial ischaemia (Berne *et al.*, 1971). Thus the mechanisms regulating the removal of adenosine from the blood are of biological importance. It has long been believed that the transience of its effects were due to inactivation by deamination in plasma, or blood or tissue cells (Clarke *et al.*, 1952); subsequent studies showed that intravenously injected adenosine was almost entirely cleared during one passage through the lungs, and that this removal was blocked by dipyridamole (Kolassa *et al.*, 1971).

We have now shown that aortic endothelial cells rapidly take up adenosine, that the high-affinity process (K_m approx. 3 μM) is potently inhibited by dipyridamole, and that intracellular adenosine is predominantly incorporated into nucleotides. Since there are over 10^{11} capillary endothelial cells in the mammalian lung our results indicate that (if aortic and pulmonary capillary endothelial cells have a similar capacity for adenosine uptake) the pulmonary endothelium could transport at least 5 μmol of adenosine/min, at a substrate concentration of 2 μM. This is more than enough to account for the observed rates of adenosine removal by the lung (Kolassa *et al.*, 1971). The concept that aortic and pulmonary endothelium may behave similarly with respect to adenosine uptake is supported by the demonstration that pulmonary endothelium and isolated aortic endothelium behave similarly with respect to two other vasoactive agents, the transport of 5-hydroxytryptamine (Shepro *et al.*, 1975; Junod & Ody, 1977; Pearson *et al.*, 1977) and the conversion of angiotensin I (Ryan *et al.*, 1976).

Some of the characteristics of adenosine uptake by endothelium have also been observed in other cells. For example, leucocytes (Taube & Berlin, 1972), lymphocytes (Strauss *et al.*, 1976), macrophages (Tsan & Berlin, 1971), hepatoma cells (Plagemann, 1971), HeLa cells (Cass & Paterson, 1977) and platelets (Sixma *et al.*, 1976) all have a high-affinity transport site for adenosine, with an apparent K_m usually less than 10 μM. A second, lower-affinity, transport site has also been found in lymphocytes (K_m approx. 400 μM) by Strauss *et al.* (1976) and in platelets (Sixma *et al.*, 1976). Although the K_m value for this site was estimated at 10 mM by Sixma *et al.* (1976), their data are better fitted by a saturable process having an apparent $K_m \leq 1$ mM, with a non-saturable influx predominating above 5 mM-adenosine.

Although the uptake of adenosine was linear with time for at least 30 min, and all our experiments used incubation times of 20 min or less, it might be argued that the characteristics of the uptake that we measured were not those of transport alone, but rather of some rate-limiting intracellular metabolic step. To demonstrate unequivocally that measured rates of uptake do indeed represent transport it is necessary to determine the uptake under conditions where the substrate tissue/medium ratio is less than 1. Unfortunately, the uptake of adenosine by endothelial cells at low (micromolar) concentrations was so rapid that such measurements were not feasible, the tissue/medium ratio exceeding 1 in about 1 s. We can, however, be confident that transport was rate-limiting, because over 90% of the incorporated adenosine was converted into nucleotides after incubation periods of up to 20 min. In addition, dipyridamole has been shown to inhibit adenosine transport (and not metabolism) in other cells (Plagemann & Richey, 1974).

To determine whether the two transport processes that we observed in endothelial cells could be distinguished other than by their kinetic characteristics, we tested several potential inhibitors and found differential effects. The lower-affinity site was preferentially inhibited by adenine, and the high-affinity site was extremely sensitive to dipyridamole. Similar selective inhibition by adenine was observed by Sixma *et al.* (1976), who did not test dipyridamole. The lack of effect of inosine on either transport site is of interest, because it suggests that both systems are relatively specific for adenosine, unlike those in erythrocytes and leucocytes, which are relatively non-specific nucleoside carriers (Plagemann & Richey, 1974).

The smooth-muscle cells of the vascular wall probably play no significant part in the removal of circulating adenosine. They are not present in capillaries (which contain most of the body's endothelial cells), their subendothelial location in larger blood vessels will minimize their exposure to circulating adenosine, and their ability to transport adenosine is poor: cultured aortic smooth-muscle cells possessed no high-affinity saturable transport process, although the effects that we observed with inhibitors suggest that a carrier-mediated process is involved. The uptake that we observed (which was poorly inhibited by dipyridamole) was less than 10% as efficient at transporting 2 μM-adenosine as the endothelial transport process. This may be a general property of smooth muscle; in preparations of stomach and intestinal smooth muscle, [^{3}H]adenosine is taken up primarily by nerve endings rather than muscle cells (Burnstock, 1975). Smooth-muscle cells do not, however, lack transport systems for purines in general: adenine, for example, is transported by similar high-affinity processes in pig aortic endothelium (de Bono *et al.*, 1976) and smooth muscle (J. D. Pearson, unpublished work).

In summary, we conclude that the efficient high-affinity transport systems for adenosine possessed by endothelial cells are probably the main route for removal of circulating adenosine, and thus that the

pulmonary endothelium is responsible for preventing adenosine released into the venous circulation from reaching peripheral arteriolar beds.

This study was supported by a grant from the Medical Research Council.

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