Br. J. Pharmac. (1983), **79**, 509–515

Metabolism and uptake of adenosine in rat isolated lung and its inhibition

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1 The metabolism of adenosine perfused through the pulmonary circulation of isolated lungs from rats was investigated radiochemically.

2 Following a 10 s infusion of radioactive [^{14}C]- or [^{3}H]-adenosine, the recovery of radioactivity in effluent from the lung after 1 min increased from 30% at 0.5 μM to 80% at 1 mM adenosine.

3 Unchanged adenosine comprised the major radioactive species in effluent, being about a third of the total up to 100 μM.

4 Uptake of radioactivity was saturable at high concentrations with an apparent *K*m of 215 μM.

5 Radioactivity retained in lung comprised over 80% as ATP and about 2% as adenosine at all concentrations.

6 Perfusion of lungs with Krebs solution containing dipyridamole (1–100 μM) or adenine (10 μM) increased the rate of radioactive efflux, decreased uptake of radioactivity by lung and decreased metabolites of adenosine (inosine and hypoxanthine) in the effluent.

7 Dipyridamole (10 μM) was more potent in decreasing uptake in guinea-pig lungs than in rat lungs.

8 From these results we conclude that the pulmonary circulation in rat lung exhibits a significant inactivation process for adenosine. The isolated lung provides a convenient preparation for studying *in situ* pharmacological or pathological modifications of this vascular inactivation process.

Introduction

Adenosine is removed on passage through the pulmonary circulation of guinea-pig and rat *in vivo* (Kolassa, Pfleger & Träm, 1971) and subsequent work has demonstrated that vascular endothelium (and smooth muscle cells) in culture take up adenosine from the culture medium (Dieterle, Ody, Ehrensberger, Stalder & Junod, 1978; Pearson, Carleton, Hutchings & Gordon, 1978). Because adenosine is anti-aggregatory (Born & Cross, 1963; Dodds, 1978), its removal from the vascular space into cells could have important consequences for platelet-vessel wall interactions and thrombosis. We have therefore undertaken a study of the fate of adenosine in isolated lungs from rats, a model in which we have already studied other metabolic reactions and uptake mechanisms of relevance to platelet behaviour (Alabaster & Bakhle, 1970; Al-Ubaidi & Bakhle, 1980; Bakhle, Lewis & Watts, 1982; Watts, Zakrzewski & Bakhle, 1982).

Methods

Preparation of lungs

Male Wistar rats (250–300 g) and Dunkin-Hartley guinea-pigs (390–420 g) were used.

Rats were anaesthetized by intraperitoneal injection of sodium pentobarbitone (60 mg/kg) and guinea-pigs killed by cervical dislocation. The lungs were removed and perfused via the pulmonary artery as described previously (Alabaster & Bakhle, 1970) with oxygenated (95% O_2:5% CO_2) Krebs solution (mM:$NaHCO_3$ 25, NaCl 120, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2 and glucose, 5.6) at 37°C, at a constant flow rate of 8 ml/min.

Infusion of radioactive adenosine

After an initial 10 min period of perfusion to clear the lung of blood and to allow for equilibration, adenosine (6.65 μCi ^{3}H or 0.665 μCi ^{14}C/infusion)

together with varying amounts of unlabelled substrate to yield the final concentration required was infused at a rate of 0.8 ml/min for 10 s into the stream of perfusate entering the pulmonary arterial cannula. Lung effluent was collected during and after this infusion either to measure total radioactivity (efflux) or for analysis by thin layer chromatography as described below.

Efflux of radioactivity

In these experiments lung effluent was collected in 15 s fractions for the first 5 min, then in 30 s fractions for the next 5 min and finally in 1 min fractions for the next 10 min. Samples (1 ml) of each fraction were taken, mixed with 10 ml of Triton-toluene scintillant (25 g PPO + 1.25 g dimethyl POPOP + 5 litres toluene + 2.5 litres Triton X-100) and the radioactivity measured.

Analysis of lung effluent

In these experiments lung effluent was collected in a single (60 s) fraction during and after the infusion of radiolabelled substrate. A further 10 s fraction was also collected to check that total radioactivity had fallen to the normally low level. The 60 s effluent fraction was acidified to pH 3.5 with 10% $HClO_4$ and 20 μl aliquots applied to plastic backed silica gel plates containing a fluorescent indicator for analysis by t.l.c. A mixture of authentic ATP, ADP, AMP, IMP, inosine, adenosine, hypoxanthine and adenine was applied to the plates to act as a carrier for the small concentration of purine present in the radioactive sample. The plates were developed in the solvent system; *n*-butanol/ethyl acetate/methanol/ammonia, 7:4:3:4 (Shimizu, Creveling & Daly, 1970). In this solvent system the nucleotides ATP, ADP, AMP and IMP remained at or near the origin while the nucleosides were better resolved with typical R_F values (for 14 cm solvent front height) of: inosine 0.26, hypoxanthine 0.46 and adenosine 0.57.

Standards were visualized under ultra-violet light and quenched purine areas were marked, cut out and transferred to scintillation vials. The purines were eluted with 1 ml distilled water for 1 h, 10 ml Triton-toluene scintillant added and the radioactivity counted in a liquid scintillation counter. Over 90% of the radioactivity applied was recovered from the plates. The mixture of labelled and unlabelled adenosine used for infusion gave the following distribution (expressed as % of the total radioactivity on the t.l.c.): inosine 3.1 ± 0.3, hypoxanthine 6.3 ± 0.3 and adenosine 80 ± 1.5 ($n = 11$).

Lung uptake Immediately following substrate infusion and effluent collection, lungs were taken down from the perfusion system, dissected free of the trachea, the remainder of the heart and any extraneous tissue, weighed and homogenized in cold 10% (w/v) trichloroacetic acid (lung weight to volume ratio, 1:10). The homogenate was centrifuged at 1,000 *g* for 20 min and the radioactivity counted in 20 μl aliquots of the supernatant. Lung supernatants were adjusted to pH 5.5 with 5 M K_2CO_3 and 20 μl samples analysed by t.l.c. in a solvent system using isobutyl alcohol/amyl alcohol/ethylene glycol monoethyl ether/ammonia/water; 45:30:90:45:60 as described by Norman, Follett & Hector (1974) giving good separation of nucleotides. Typical R_F values with a solvent front of 10 cm were: ATP 0.15, ADP 0.32, IMP 0.39, AMP 0.48, inosine 0.64, adenosine + hypoxanthine (not resolved in this solvent) 0.75 and adenine, 0.84. To check for breakdown during the extraction process, [^{3}H]-ADP, -AMP, -ATP and -adenosine were added separately to perfused lungs immediately before homogenization in trichloroacetic acid and the extraction and analysis carried out as usual. Over 93% of each compound remained unchanged after extraction and analysis.

Inhibitor treatment of perfused lung In the experiments involving dipyridamole and adenine, the lungs were perfused with Krebs solution containing the required concentration of the inhibitor for 20 min before and during the infusion of substrate and effluent collection. The control lungs in this series of experiments were perfused with Krebs solution alone over the same time.

Statistical methods

Results are given as the mean (±s.e.) value from the number of experiments shown, each experiment representing a different lung. The statistical significance of the difference between mean values was estimated by Student's *t* test for unpaired samples and a value of $2P < 0.05$ was taken as indicating a significant difference.

Materials

Sodium pentobarbitone (Sagatal) was purchased from May and Baker Ltd. The following compounds were purchased from Sigma: adenosine 5'-diphosphate sodium salt; adenosine 5'-monophosphoric acid sodium salt; adenosine 5'-triphosphate disodium salt; inosine 5'-monophosphoric acid sodium salt; adenosine; adenine; inosine; hypoxanthine. Dipyridamole was a generous gift from Boehringer Ingelheim. The radiolabelled substrate was purchased from The Radiochemical Centre, Amersham: [2-^{3}H]-adenosine (21 Ci/mmol) and [8-^{14}C]-adenosine

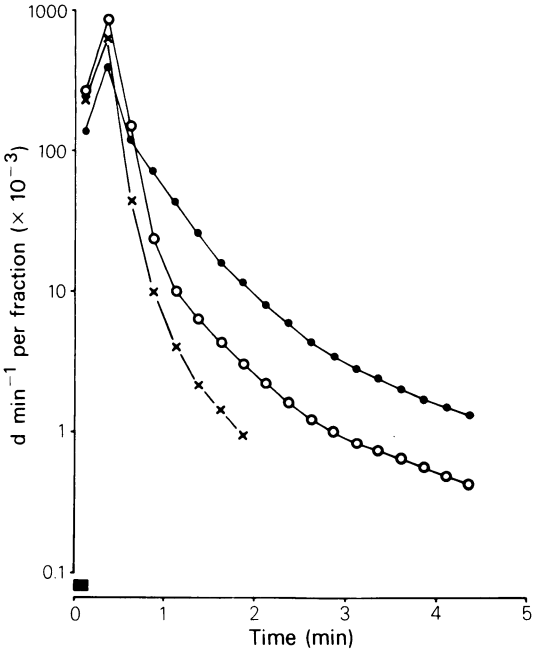

Figure 1 Efflux of radioactivity from rat isolated lung, following infusions of [^{14}C]-adenosine. Substrate (0.665 μCi) at the concentrations shown was infused for 10 s (black bar at foot of Figure) and effluent collected in 15 s fractions for 4.5 min. Efflux of radioactivity was rapid (note that the ordinate is on a logarithmic scale) at 10 μM (●) and faster at 1 mM (○) adenosine. For comparison, the efflux following 10 μM ADP (×) infused in the same way is also shown.

(57 mCi/mmol). All solvents used were of analytical grade and purchased from BDH Chemicals Ltd. Plastic backed t.l.c. plates of silica gel with fluorescent indicator (20 cm × 20 cm) were obtained from Merck.

Results

Efflux of radioactivity

The appearance of radioactivity in lung effluent following infusion of [^{3}H]-adenosine into the pulmonary circulation of rat isolated lung is illustrated in Figure 1. Although collections were made for 15 min, only the results for the first 5 min have been shown. The efflux profile for ADP has been taken from our previous work (Chelliah & Bakhle, 1983. Following [^{3}H]-adenosine infusions, efflux of ^{3}H was rapid; at 10 μM (the uppermost curve), 90% of the ^{3}H appearing in 15 min had been collected in the first minute's fractions. Efflux was also related to the concentration of substrate, radioactivity appearing in effluent more rapidly at 1 mM than at 10 μM. However, even at 1 mM adenosine, efflux of radioactivity was still slower than that following infusion of ADP (10 μM; the lowest curve).

Analysis of effluent radioactivity

Effluent collected for the first minute was analysed by t.l.c. using a solvent system designed to give good resolution of non-phosphorylated derivatives (Figure 2). The major product at 0.5 μM or at 1 mM (Figure 2) and at intermediate concentrations (Table 1) was unchanged substrate. Although adenosine comprised a constant proportion of effluent radioactivity from 0.5 μM to 100 μM, the proportions of inosine and hypoxanthine increased and that of the phosphorylated compounds decreased gradually

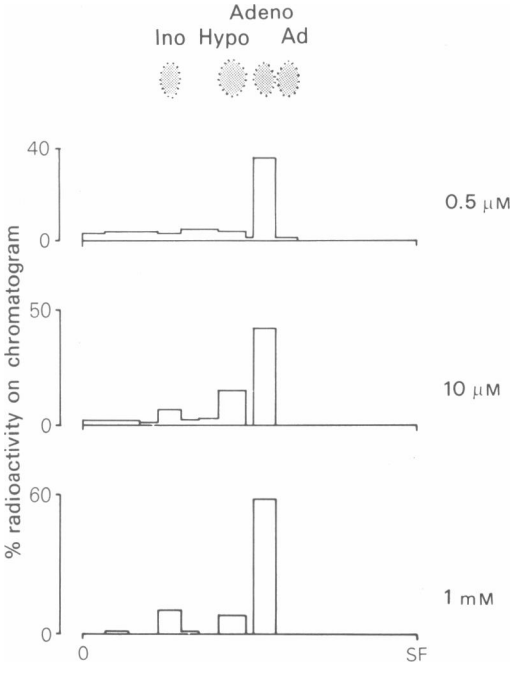

Figure 2 Chromatographic analysis of effluent from rat lung after infusion of adenosine. At the top of the Figure, the positions of the marker compounds are shown and at the foot of the Figure, 0 = origin and SF = solvent front for the t.l.c. plate. The 1 min effluent following infusion of [^{3}H]-adenosine at the concentrations shown was analysed and each t.l.c. trace shows the distribution of radioactivity on the t.l.c. plate for a single experiment. Unchanged adenosine (Adeno) was always the major component with hypoxanthine (Hypo) and inosine (Ino) as the main metabolites. Note the low radioactivity associated with the origin where phosphorylated derivatives would be found.

Table 1 Analysis of effluent radioactivity following [^{3}H]-adenosine infusions to rat isolated lung

Concentration (no. of lungs)	*Total effluent radioactivity*	*Distribution of effluent radioactivity*			
		Nucleotides	*Inosine*	*Hypoxanthine*	*Adenosine*
0.5 μM (3)	30±4	6±1	8±1	14±1	38±7
1 μM (3)	37±4	6±1	8±1	12±1	45±6
3 μM (3)	35±4	6	10	16±1	38±3
10 μM (6)	48±4	4	10	19±1	44±2
30 μM (3)	56±3	4	12	23±1	38±2
100 μM (3)	64±5	3	13±3	24±4	41±8
1 mM (6)	84±2	2	11±1	10±1	63±1

Adenosine at different concentrations (containing $\approx 1.3 \times 10^7$ d min^{-1} ^{3}H) was infused for 10 s into the pulmonary circulation and effluent collected during the infusion and for 50 s afterwards to yield the first minute fraction. Hence total effluent radioactivity refers to the total ^{3}H in the first minute fraction expressed as a percentage of the total radioactivity infused; distribution of effluent radioactivity between the various components is expressed as a percentage of the total radioactivity in the first minute fraction. The values in the table are the means (±s.e.mean) of results from the number of lungs shown. Where the standard errors were less than 1%, they have been omitted.

over the same range. At the highest concentration (1 mM), the adenosine component was much higher and the inosine and hypoxanthine components decreased in proportion.

Because lung effluent had been shown to exhibit ADP-ase activity (Chelliah & Bakhle, 1983), in three experiments adenosine (10 μM and 1 mM) was incubated with samples of effluent collected from lung and with Krebs solution which had not been perfused through lung, for up to 4 h at 37°C. At 1 mM, 94±1.0% and at 10 μM, 84±0.3% of the adenosine was recovered unchanged after 4 h incubation with lung effluent compared with 95% recovery after an equivalent incubation with Krebs solution alone.

Radioactivity in lung

As shown in Table 1, the proportion of the infused radioactivity appearing in effluent was related to the concentration of substrate, exceeding 50% at 30 μM and higher. The remainder of the radioactivity was found in lung (at the end of the first minute's collection) associated with the supernatant fraction after precipitation with trichloracetic acid. The pellet contained less than 10% of the ^{3}H infused. Analysis of the supernatant radioactivity showed it to comprise mainly (>80%) ATP (Table 2) at all concentrations from 0.5 μM to 100 μM adenosine. At 1 mM the amounts of ^{3}H in lung were too small to analyse although the total could be measured.

Assuming that all the radioactivity in lung entered as adenosine, we assessed the concentration-dependence of adenosine uptake in perfused lung (Figure 3). Uptake was not linearly related to substrate concentration but neither was uptake obviously saturated up to 100 μM. Analysis of the results from the whole range of concentrations from 0.5 μM to 1 mM by the Eadie-Hofstee transformation gave values of apparent Km 215 μM and of V_{max} 157 nmol min^{-1} per lung.

Table 2 Analysis of radioactivity retained in lung following [^{3}H]-adenosine infusion to rat isolated lung

Concentration of adenosine (μM)	*Total lung radioactivity*	*Distribution of lung radioactivity*					
		ATP	*ADP*	*IMP*	*AMP*	*Inosine*	*Adenosine + Hypoxanthine*
0.5	64±4	86±1	7	1	3±1	1	2
1	57±4	87±1	7	1	2	1	2
3	54±1	88±1	6	1	2	1	2
30	41±4	84±1	7	2	3	1	2
100	28±2	85±1	7	2	2	1	2

The values in the table are the means (±s.e.mean, except where the s.e. was less than 1% and has been omitted) of results from 3 lungs at each concentration tested. As in the previous table, 'total lung radioactivity' is expressed as a percentage of the total radioactivity infused and distribution of lung radioactivity as a percentage of the total lung radioactivity. Immediately after the collection of the first minute fraction the lungs were analysed by homogenization in trichloracetic acid and chromatography.

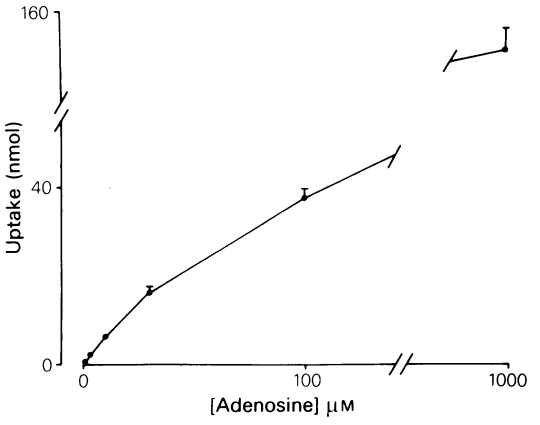

Figure 3 Variation of the uptake of adenosine by rat isolated lung with substrate concentration in the perfusate. Uptake of adenosine was measured as uptake of radioactivity (after 1 min) in at least 3 lungs at each concentration from 0.5 μM to 1 mM. The points shown represent mean values (with s.e. shown where these are larger than the symbols) at each concentration; $n = 3-6$. Both axes are linear with a break between 100 μM and 1 mM. Uptake of adenosine was saturable over a wide concentration range.

Effects of dipyridamole and adenine

Dipyridamole and adenine, known to inhibit adenosine uptake in cultured cell systems (Dieterle *et al.*, 1978; Pearson *et al.*, 1978) were investigated in rat and guinea-pig perfused lung. The lungs were perfused with Krebs solution containing the inhibitors for 20 min before and during the substrate infusion and collection. In rat lung, both compounds increased the efflux of radioactivity following [^{3}H]-adenosine infusion (Table 3). A moderate but significant increase in radioactive efflux was observed over the first 30 s of collection but not in the subsequent two 30 s periods. Thus, the increased radioactivity collected over a 20 min period in the presence of the inhibitors appeared to be accounted for by the increase in the initial 30 s collection.

A further analysis of the effects of dipyridamole over a range of concentrations on the disposition of [^{3}H]-adenosine infused at 10 μM (Table 4) showed that inhibition of ^{3}H retention by the lung was significant only at 10 and 100 μM dipyridamole, giving 27 ± 3 and $55 \pm 2\%$ inhibition respectively. This effect was accompanied by an increase in effluent radioactivity and a decrease in the metabolites of adenosine in the effluent. Adenine was more potent than dipyridamole giving $47 \pm 2\%$ inhibition of ^{3}H retention at 10 μM, but here the metabolites of adenosine in effluent were much less affected.

In guinea-pig lungs, although the normal uptake and metabolism of [^{3}H]-adenosine was largely similar to that observed in rat lungs, the effects of dipyridamole were more marked. Thus, 10 μM dipyridamole caused $76 \pm 2\%$ inhibition of uptake in guinea-pig lungs, nearly three times the effect caused by the same concentration and exceeding the inhibition caused by 100 μM dipyridamole in rat lungs.

Discussion

In our experiments the rat perfused lung was capable of removing adenosine from the vascular space by a process of uptake and metabolism. As its metabolites were either inactive (hypoxanthine and inosine) or else retained in lung tissue (adenine nucleotides), this removal was equivalent to an inactivation process for the biologically active adenosine. Net survival of adenosine on a single passage was between 10–18% from 0.5–3 μM and increased to just over 20% at 100 μM, so that the inactivation of adenosine in the pulmonary circulation was extensive and comparable with that of prostaglandin E_2 (PGE_2) or 5-hydroxytryptamine (5-HT: Bakhle & Vane, 1974).

There were several indications that metabolic transformation of adenosine, like that of PGE_2 and 5-HT, followed uptake by lung cells. Firstly, the efflux of radioactivity in lung effluent following infusion of [^{3}H]-adenosine, while being rapid, was still slower than that following infusion of [^{3}H]-ADP. This was expected since ADP, which is metabolized

Table 3 Efflux of radioactivity from rat isolated lung following infusion of [^{3}H]-adenosine (10 μM) in the presence of inhibitors of adenosine uptake

	Effluent radioactivity in each collection period			
	0–30 s	*30–60 s*	*60–90 s*	*Total: 0–20 min*
Control (6)	40 ± 3	11 ± 2	2 ± 1	57 ± 1
Dipyridamole 10 μM (3)	51 ± 2*	10 ± 3	1	65 ± 3*
Adenine 10 μM (3)	57 ± 1*	13 ± 4	1	73 ± 5*

The values in the table are the mean (±s.e.mean) of results from the number of lungs shown for each treatment. Results are expressed as a percentage of the total ^{3}H infused ($\approx 1.3 \times 10^7$ d min^{-1}).
*Significantly different from control value ($2P < 0.05$).

Table 4 Effect of dipyridamole and adenine on the distribution of radioactivity derived from [^{3}H]-adenosine infusions in lung and effluent

	Total radioactivity in		*Distribution of effluent radioactivity*		
	Lung	*Effluent*	*Inosine*	*Hypoxanthine*	*Adenosine*
(a) Rat lungs					
Control (6)	48±2	48±4	10	19±1	44±2
Dipyridamole 1 μM (3)	39±3	54±3	9	17	51±1*
(3) 10 μM	30±3*	62±3*	7±1*	12±1*	63±3*
(3) 100 μM	19±3*	67±3*	6±1*	8*	71±1*
Adenine 10 μM	20;25	74;64	8;7	23;26	54;50
(b) Guinea-pig lungs					
Control (3)	30±2	59±3	7	11±1	63±2
Dipyridamole 10 μM (3)	5±2*	82±3*	5*	6*	75±1*

The values in the table are the means (± s.e.mean) of results from the number of lungs shown. Where standard errors are less than 1% they have been omitted. In all cases, [^{3}H]-adenosine was infused to give a final concentration of 10 μM and about 1.3×10^7 d min^{-1} per infusion. 'Total radioactivity' is expressed as a percentage of infused radioactivity and 'Distribution of effluent radioactivity' as a percentage of the total ^{3}H in the 1 min effluent collected. *Significantly different from control value.

extensively to AMP (Chelliah & Bakhle, 1983) by membrane-bound enzymes, has a pulmonary transit time comparable with that of the vascular marker [^{14}C]-dextran (Crutchley, Eling & Anderson, 1978) because both substrate and product are nucleotides and thus excluded from cells. Adenosine, on the other hand, is known to be taken up by endothelial and smooth muscle cells where it may be attacked by intracellular adenosine deaminase and nucleoside phosphorylase (Rubio, Wiedmeier & Berne, 1972) to yield inosine and hypoxanthine respectively and by kinases to form adenine nucleotides (Pearson *et al.*, 1978). The non-phosphorylated products must have left the cells rapidly so that, overall, the radioactive efflux was over in a few minutes. If these two enzymes had been extracellular, the efflux of ^{3}H following adenosine infusions should have been comparable with that following ADP infusion. Furthermore, extracellular adenosine deaminase is usually found in blood (Conway & Cooke, 1939) and this should have been washed out during the perfusion.

Another indication that uptake precedes metabolism to inosine and hypoxanthine was the decrease in these metabolites when lungs were treated with dipyridamole, a known inhibitor of uptake in other systems (Kübler & Bretschnider, 1964; Kolassa *et al.*, 1971; Dieterle *et al.*, 1978; Pearson *et al.*, 1978). Once taken up, most of the adenosine was phosphorylated to ATP and thus retained and a smaller amount re-entered the vascular space as the inosine and hypoxanthine metabolites. It is thus most likely that in our experiments, adenosine surviving passage through the pulmonary circulation had never left the vascular space.

Assuming that radioactivity is taken up by lung tissue only as adenosine, we found that the uptake of adenosine in rat lung was a saturable process with an apparent *K*m value of 215 μM. Our value of *K*m is within the range of the low affinity process, *K*m of 250 μM and 0.3–1.1 mM, described for endothelial cells of the pig by both Pearson *et al.* (1978) and Dieterle *et al.* (1978) respectively. However, both groups of workers also reported a high affinity uptake process (*K*m 3 μM) which was not detectable in our system.

In our experiments, as expected from the results of others (Kolassa *et al.*, 1971; Dieterle *et al.*, 1978; Pearson *et al.*, 1978) dipyridamole induced a concentration-related inhibition of adenosine uptake, an equimolar ratio of substrate and inhibitor giving about 25% inhibition. We were also able to confirm that this inhibition was more effective in guinea-pig isolated lungs than in rat isolated lungs as described by Kolassa *et al.* (1971) in their studies *in vivo*. The efficacy of adenine as an inhibitor of adenosine uptake in rat lung agrees with the results of Pearson *et al.* (1978) in showing greater potency than dipyridamole.

It appears therefore that in whole perfused lung, adenosine is extensively inactivated by uptake and metabolism and that this observed uptake is compatible with the presence of uptake systems demonstrable in pure cultures of endothelial cells and smooth muscle cells. The existence of such an extensive

inactivation process must be taken into account when postulating roles for adenosine as a pharmacological mediator.

Adenosine has been proposed as a local hormone with wide-ranging biochemical and physiological activity on both vascular and non-vascular smooth muscle, nervous tissue and the heart, lipolysis and glucose oxidation in adipose tissue, (Arch & Newsholme, 1978) as well as on platelet aggregation. Although adenosine is a potent anti-aggregatory agent for platelets from man and many other species (Born & Cross, 1963), in the rat its role in aggregation is not so clear. While adenosine has been reported to have no effect on aggregation in rat platelet-rich plasma (Cuthbertson & Mills, 1963), Constantine (1967) found that the anti-aggregatory activity of adenosine depended on how the rat platelets were prepared. Furthermore, Born & Philp (1965) found that, *in vivo*, adenosine is capable of inhibiting thrombus formation in the rat. For adenosine to play the regulatory role proposed, its concentration in blood must be controlled and uptake of adenosine by endothelial cells may provide the most important control mechanism (Pearson *et al.*, 1978).

The isolated perfused lung preparation provides a large, easily accessible endothelial cell surface in which anatomical relationships within the whole organ are maintained, in the absence of blood cells and enzymes. It also provides an excellent model in which to study the pharmacological or pathological modifications of the removal of adenosine, modifications which could have important consequences for the maintenance of vascular tone and for platelet-vessel wall interactions.

This work and one of us (RC) was generously supported by the Wellcome Trust.

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(Received November 15, 1982.
Revised January 10, 1983.)