Biochemical Pharmacology, Vol. 20, pp. 3359–3365. Pergamon Press, 1971. Printed in Great Britain

A SPECIES DIFFERENCE IN THE UPTAKE OF ADENOSINE BY HEART

SONDRA V. HOPKINS and R. G. GOLDIE
Victorian College of Pharmacy, 381 Royal Parade, Parkville, Victoria 3052, Australia

(*Received* 24 *March* 1971; *accepted* 16 *June* 1971)

Abstract—[8-^{14}C]Adenosine was taken up by hearts isolated from rats or guinea-pigs, and was retained mainly in the form of its phosphate derivatives, but the kinetics of the uptake processes differed in each species. Dipyridamole blocked uptake of adenosine in the guinea-pig heart by inhibiting its phosphorylation. Soluble adenosine kinase was not inhibited by dipyridamole, so presumably the dipyridamole-sensitive enzyme was membrane-bound. Rat hearts took up less adenosine than guinea-pig hearts at 32°–37°, and the uptake process in rats was not blocked by dipyridamole. A possible explanation, consistent with these findings, is that the rat heart lacks the membrane-bound enzyme, adenosine uptake depending upon a soluble kinase.

IT HAS been known for many years that adenosine dilates the coronary arteries.[1–3] Berne[4] and Gerlach and Deuticke[5] have suggested that dilation of the coronary vessels by dipyridamole[6,7] may be the result of an increase in the availability of endogenous adenosine.

Dipyridamole augments adenosine-induced heart-block in the guinea-pig,[8] presumably by reducing the uptake of adenosine into cardiac tissues.[9,10] However, in the rat heart, adenosine is not potentiated by dipyridamole.[8]

It therefore seemed probable that dipyridamole would not block uptake of adenosine into rat heart and, furthermore, that the mechanisms of adenosine uptake and retention by rat heart would differ from those of the guinea-pig. The experiments described below were designed to test this idea.

METHODS

Animals used were of either sex. Rats were of the Glaxo Wistar strain and weighed between 200 and 250 g. Guinea-pigs weighed between 250 and 300 g.

Uptake of [8-^{14}C]*adenosine.* Hearts were isolated and perfused via an aortic cannula with McEwen's[11] solution, saturated with 5% CO_2 in O_2. The temperature of the perfusion fluid was 32° and the flow rate was maintained at 6·5 ml/min (unless otherwise stated in Results) by a Watson–Marlow peristaltic pump. In some experiments, dipyridamole (5×10^{-6} M) was added to the perfusion fluid. After 5–10 min, [8-^{14}C]adenosine (2·5–102·5 nmoles, 70 nc) was injected over a period of 1 min in five 100 μl lots. One min later, after most of the radioactivity had disappeared from the perfusate, the heart was removed from the cannula, weighed, and homogenized in 6% trichloracetic acid. The radioactivity in the acid supernatant was estimated by liquid scintillation counting.[12]

Metabolism of [8-^{14}C]*adenosine.* In some of the experiments with perfused hearts, the chemical nature of the ^{14}C retained by the ventricles was determined after the

injection of [8-^{14}C]adenosine (2·5 nmoles, 70 nc). Conditions for perfusion of the hearts are described above. One minute after the injection of [8-^{14}C]adenosine, each ventricle was removed, weighed, and homogenized in 3 vol. 0·5 M perchloric acid. All operations were carried out at 0–4°. After centrifugation, the supernatant was neutralised with 2 M KOH. The mixture was again centrifuged and 100 μl was streaked in 2 μl quantities onto a glass plate coated with Machery Nagel cellulose MN300. The plate was developed in *n*-butanol–methanol–H_2O–NH_3 (60 : 20 : 20 : 1) as recommended by Randerath.[13] Adenosine, AMP, ADP, ATP, inosine and hypoxanthine were used as markers. When the plate was dry, the positions of the markers were observed under u.v. light. Cellulose was scraped from the plate to correspond to each of the markers, the phosphates being collected together. The radioactivity was estimated by counting the scrapings distributed in 0·4% 2,5-bis-2-(5-tertbutylbenzoxazolyl)-thiophene (BBOT, Packard) in toluene to which had been added 4% Thixotropic-gel Packard).

The effect of dipyridamole on the metabolism of [8-^{14}C]*adenosine* was measured in guinea-pig heart. The heart was collected, washed, and cut into fine slices with a scalpel blade. Slices (200 mg) were incubated with and without dipyridamole (5×10^{-6} M) in 2 ml of McEwen's[11] solution containing [8-^{14}C]adenosine (32 nmoles, 900 nc), and gassed with 5% CO_2 in O_2. After 10 min, the muscle was collected and treated as described above. The purine derivatives in the incubation medium were extracted onto 10 mg of powdered charcoal according to Jacob and Berne.[14] After 4 hr shaking, the supernatant was decanted and the charcoal was washed with 1 ml of distilled H_2O. The radioactive material was extracted from the charcoal into 1 ml of 50% aqueous ethanol, pH 10. Separation by thin layer chromatography was as described above.

A cell-free homogenate was prepared by pooling four guinea-pig hearts and homogenizing in 4 vol. of 0·154 M KCl in a Braun blender at 4°. Further cell disruption was achieved by homogenization using a Thomas glass homogenizer with a motor driven teflon pestle. The homogenate was centrifuged at 5000 *g* for 15 min, the supernatant being collected and centrifuged for 1 hr at 100,000 *g*. An incubation medium[15] with or without dipyridamole was prepared, having the following constituents in 1·6 ml; [8-^{14}C]adenosine, 80 nmoles, 220 nc; ATP, 14 nmoles; potassium 3-phosphoglycerate, 12 nmoles; tris-HCl, pH 7·5, 80 nmoles; and KCl, 250 nmoles. To either solution was added 0·2 ml of the 100,000 *g* supernatant and the mixtures were incubated for 1 hr at 37° with shaking. Samples of 0·3 ml were collected at 1, 2, 8, 20, 40 and 60 min, to which were added 0·2 ml 0·5 M perchloric acid. After neutralization with 2 M KOH, the procedure as described above for the separation of the radioactive components was followed.

Materials. [8-^{14}C]Adenosine (28 mc/m-mole, The Radiochemical Centre, Amersham), dipyridamole (2,6-bis-(diethanolamine)-4,8-dipiperidino-pyrimido-(5,4-*d*)-pyrimidine as Persantin, Boehringer Ingelheim), adenosine, inosine, hypoxanthine, AMP, ADP, ATP, potassium 3-phosphoglycerate, (Sigma Chemical Company).

RESULTS

Dipyridamole on adenosine uptake by rat heart. After injection of [8-^{14}C]adenosine (2·5 nmoles) into the perfusion fluid (flow rate, 4 ml/min), the radioactivity retained

TABLE 1. THE CHEMICAL DISTRIBUTION OF ^{14}C IN PERFUSED HEARTS AFTER INJECTION OF [8-^{14}C]ADENOSINE

	Phosphates	Inosine	Hypoxanthine	Adenosine*
Rat	76·0 ± 3·8	10·7 ± 0·9	3·5 ± 1·5	9·8 ± 1·9
Guinea-pig	83·1 ± 3·9	12·3 ± 2·7	1·6 ± 0·7	3·4 ± 1·1

[8-^{14}C]Adenosine (2·5 nmoles) was injected into the perfusion fluid over a period of 1 min. One min later, hearts were removed, and prepared for analysis of adenosine and its metabolites. Results are expressed as percentages of the total ^{14}C retained by the hearts. Each result is the mean, with S.E., of five observations.

* A significant difference between the two species was observed ($0·02 < P < 0·05$).

by rat isolated hearts was equivalent to 0·90 ± 0·13 nmoles adenosine/g (mean ± S.E. of five observations). When dipyridamole (5 ± 10^{-6} M) was present in the perfusion fluid, uptake of radioactive adenosine by the hearts remained unchanged (0·90 ± 0·07 nmoles/g). A similar concentration of dipyridamole virtually abolished adenosine uptake in guinea-pig isolated hearts.[10]

Adenosine metabolism in rat and guinea-pig hearts. Analysis of the chemical nature of the radioactivity retained by perfused hearts after injection of [8-^{14}C]adenosine is shown in Table 1. In both rat and guinea-pig hearts, most of the [8-^{14}C]adenosine retained was in the form of phosphate derivatives. Metabolism of adenosine was similar in both species, the only marked difference being the greater proportion of unchanged adenosine remaining in the rat heart.

Concentration dependence. In rat or guinea-pig perfused hearts, increasing the concentration of unlabeled adenosine administered in the presence of a constant dose of

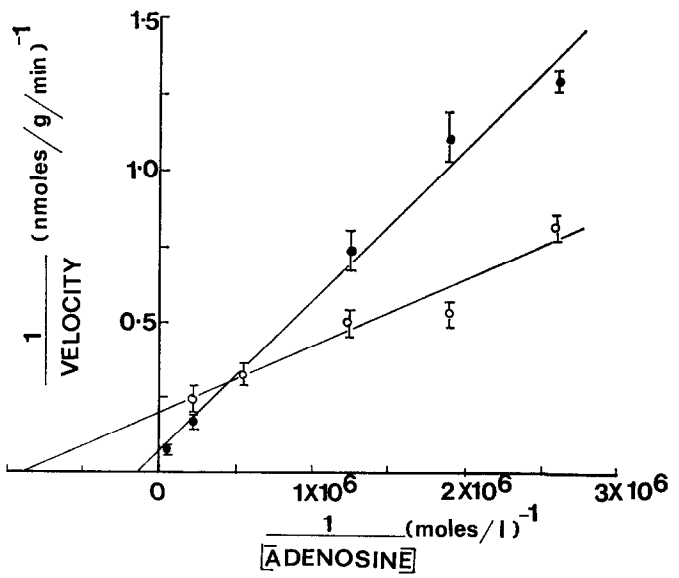

FIG. 1. A double reciprocal plot of the uptake of [8-^{14}C]adenosine by perfused guinea-pig (○——○) or rat heart (●——●). Each point is the mean of five observations, and the S.E. is indicated by the bar.

[8-^{14}C]adenosine reduced the amount of the labeled nucleoside taken up. Total uptake of adenosine was calculated for each adenosine concentration and the results are presented in the form of a double reciprocal plot of the uptake of adenosine by the perfused heart (Fig. 1). For rat heart a Michaelis constant (K_m) of $5{\cdot}0 \times 10^{-6}$ M, and a maximum velocity (V) of 12 nmoles.g^{-1}.min^{-1} were obtained by the method of Lineweaver and Burk.[16] For guinea-pig heart K_m was $1{\cdot}0 \times 10^{-6}$ M and V was 4·5 nmoles.g^{-1}.min.

Temperature dependence. Uptake of adenosine by rat or guinea-pig hearts was decreased by reducing the temperature of the perfusion fluid (Fig. 2). In rat heart, uptake was maximal at 32°. However, in guinea-pig heart, uptake was significantly greater at 37° than at 32°. The effect of changes in temperature on adenosine uptake was more marked in the guinea-pig heart than in the rat heart.

Dipyridamole and the metabolism of [8-^{14}C]*adenosine.* When guinea-pig heart slices were incubated in a medium containing [8-^{14}C]adenosine ($1{\cdot}6 \times 10^{-5}$ M, 450 μc/l.) for 10 min, most of the adenosine was deaminated, and some phosphorylated (Table 2). Dipyridamole (5×10^{-6} M) significantly reduced the amount of phosphorylated derivatives after incubation of guinea-pig heart slices; no effect was seen, however, on the levels of deaminated derivatives formed (Table 2)

Analysis of the incubation media showed that approximately 10% of the [8-^{14}C]-adenosine remained unchanged. There was about three times as much inosine as hypoxanthine in the incubation media, a proportion similar to their concentration ratio in the heart slices. However, no phosphorylated derivatives could be found in the incubation media. The concentration of deaminated derivatives (inosine plus hypoxanthine) in the incubation media was estimated to be 14 nmoles/ml, after correction for the proportion not absorbed onto charcoal. Assuming that the concentra-

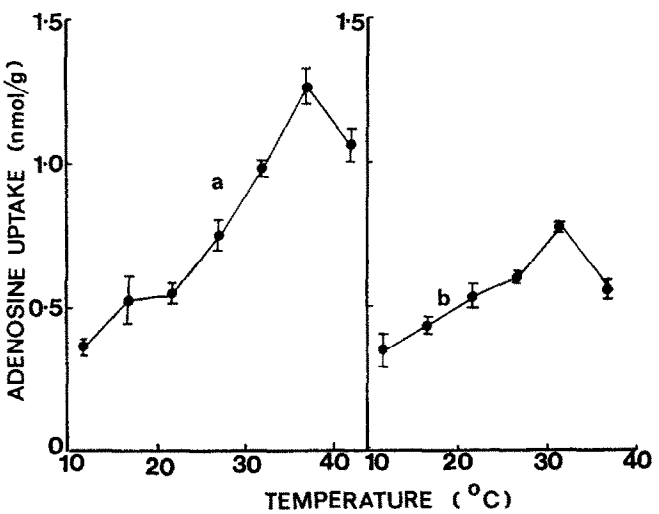

FIG. 2. The effect of change in temperature of the perfusion fluid on the uptake of adenosine in (a) guinea-pig heart and (b) rat heart. [8-^{14}C]Adenosine (2·5 moles, 70 nc) was injected. The point at 37° for rat heart is the mean of eight observations, all other points are the means of four observations, the standard errors are shown in each case.
A significant difference was observed between the uptake at 32° and 37° in guinea-pig or rat heart ($0{\cdot}001 < P < 0{\cdot}01$).

TABLE 2. THE CHEMICAL DISTRIBUTION OF ^{14}C IN GUINEA-PIG HEART SLICES AFTER INCUBATION WITH [8-^{14}C]ADENOSINE

	Phosphates*	Inosine	Hypoxanthine
Control	0·69 ± 0·03	3·31 ± 0·38	1·23 ± 0·26
Dipyridamole 5×10^{-6} M	0·45 ± 0·01	2·83 ± 0·34	0·92 ± 0·08

The effect of dipyridamole on the distribution of ^{14}C in heart slices incubated for 10 min with [8-^{14}C]adenosine ($1·6 \times 10^{-5}$ M, 450 nc/ml). Results are expressed as nmoles/g of tissue. Each result is the mean, with S.E., of four observations.

* A significant difference between control and dipyridamole-treated tissues was observed ($0·01 < P < 0·02$).

tions of inosine and hypoxanthine in the extracellular fluid of the heart slices were in equilibrium with those in the incubation media, and that the extracellular water in cardiac tissue is 0·16 ml/g,[17] then it appears that about 60 per cent of the inosine and hypoxanthine were extracellular. These results suggest that deamination occurred extracellularly.

Dipyridamole did not affect the rate of phosphorylation or deamination of [8-^{14}C]-adenosine when incubated with a particulate-free homogenate from guinea-pig heart

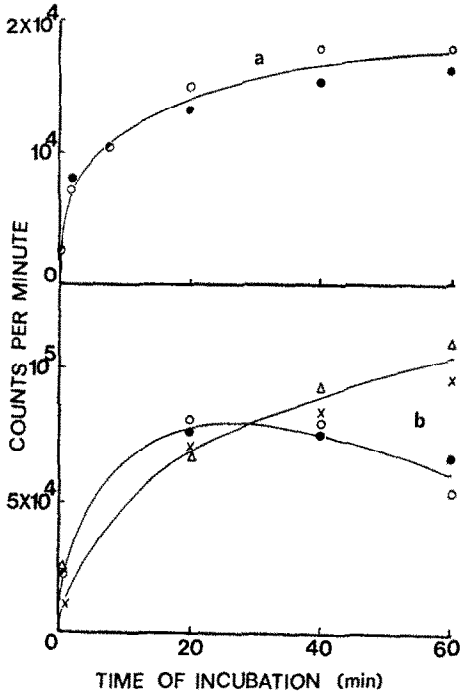

FIG. 3. The effect of 5×10^{-6} M dipyridamole on the metabolism of [8-^{14}C]adenosine (5×10^{-5} M, 28 mc/m-mole) in an incubation medium containing a sample of a 100,000 *g* supernatant from guinea-pig heart. (a) The phosphorylation of adenosine with ○——○ and without ●——● dipyridamole. (b) The deamination of adenosine: with dipyridamole, inosine ○——○, hypoxanthine △——△; without dipyridamole, inosine ●——●, hypoxanthine ×——×. Count rates are uncorrected for quenching.

(Fig. 3). During the initial 10-min period of incubation the rates of phosphorylation and deamination were approximately 25 nmoles/min/g of tissue and 250 nmoles/min/g of tissue respectively.

DISCUSSION

In marked contrast to the block of adenosine uptake into guinea-pig hearts by dipyridamole (5×10^{-6} M),[9,10] uptake into rat hearts was unaffected by dipyridamole. This observation accounts for the inability of dipyridamole to potentiate adenosine in rat heart.[8] It also adds support to the concept that dipyridamole potentiates adenosine by blocking its uptake.

Uptake and retention of adenosine by rat heart appear to involve mechanisms that differ from those in guinea-pig heart. In the rat, as in the guinea-pig, adenosine uptake is an active process, since competition of adenosine molecules for uptake was observed (Fig. 1). A K_m of $5{\cdot}0 \times 10^{-6}$ M and V of 12 nmoles/g/min in rat heart differ from the corresponding values for guinea-pig heart, viz. $1{\cdot}0 \times 10^{-6}$M and 4·5 nmoles/g/min. The considerably greater uptake of adenosine by guinea-pig heart (Fig. 2) suggests the presence of a more efficient mechanism for the capture of adenosine in this species. That different mechanisms are involved is also suggested by the different temperature optima observed in the hearts of the two species (Fig. 2).

The idea that dipyridamole potentiates adenosine by blocking its inactivation by adenosine deaminase has been proposed and discussed by various authors.[18–21] Direct evidence in favour of this idea arises from relatively few experiments in which remarkably high concentrations ($> 5 \times 10^{-5}$ M) of the drug were used.[18,20] The experiments described in this paper (Table 2, Fig. 3) reveal that dipyridamole (5×10^{-6} M) does not inhibit adenosine deaminase from guinea-pig heart. The results suggesting that deamination of [8-^{14}C]adenosine occurred extracellularly are probably explained by the release of adenosine deaminase from the damaged cells. Loss of adenosine deaminase from isolated cat heart into the perfusion fluid was observed by Jacob and Berne.[14]

Jacob and Berne,[14] found that adenosine was retained mainly in the form of its phosphate derivatives. The present results show that is also true in guinea-pig and rat hearts (Table 1). Berne[4] suggested that this phosphorylation of adenosine occurred within the myocardial cell membrane. The experiments with guinea-pig heart slices showed that the phosphorylation of adenosine was either membranal or intracellular, since no radioactive adenosine phosphates were detected in the incubation medium. Because dipyridamole reduced the amount of phosphorylated adenosine derivatives found in the heart slices (Table 2) while not inhibiting the soluble kinase (Fig. 3), it is concluded that uptake of adenosine by guinea-pig heart involves a membrane-bound kinase. It is this enzyme in the cell membrane that is inhibited by dipyridamole.

It is possible that the rat heart lacks a membranal adenosine kinase, and that phosphorylation of adenosine is catalyzed by a soluble intracellular enzyme, which, like that in the guinea-pig heart, is resistant to dipyridamole.

Acknowledgements—We thank Dr. Anne Stafford for helpful advice, Dr. K. Higgins for his gift of dipyridamole and the Staff of the Department of Medicine, Royal Melbourne Hospital for making available their scintillation counter.

REFERENCES

1. A. N. Drury and A. Szent-Györgyi, *J. Physiol., Lond.* **68,** 213 (1929–30).
2. K. Zift, *Arch. exp. Path. Pharmak.* **149,** 76 (1930).
3. A. M. Wedd, *J. Pharmac. exp. Ther.* **41,** 355 (1931).
4. R. M. Berne, *Am. J. Physiol.* **204,** 317 (1963).
5. E. Gerlach and B. Deuticke, *Arzneimittel-Forsch.* **13,** 48 (1963).
6. Von H. G. Menge and R. Kadatz, *Arzneimittel-Forsch.* **9,** 476 (1959).
7. A. Jageneau and W. Schaper, *Arzneimittel-Forsch.* **17,** 582 (1967).
8. A. Stafford, *Br. J. Pharmac.* **28,** 218 (1966).
9. N. Kolassa, K. Pfleger and W. Rummel, *Eur. J. Pharmac.* **9,** 265 (1970).
10. Von I. Volkmyer and K. Pfleger, *Naunyn Schmiedebergs Arch. Pharmak. exp. Path.* **263,** 155 (1969).
11. L. M. McEwen, *J. Physiol., Lond.* **131,** 678 (1956).
12. G. A. Bray, *Anal. Biochem.* **1,** 279 (1960).
13. K. Randerath, *Nature, Lond.* **205,** 908 (1965).
14. M. I. Jacob and R. M. Berne, *Am. J. Physiol.* **198,** 322 (1960).
15. D. H. Ives, P. A. Morse and V. R. Potter, *J. biol. Chem.* **238,** 1467 (1963).
16. W. Lineweaver and D. Burk, *J. Am. chem. Soc.* **56,** 658 (1934).
17. D. M. Woodbury, in *Physiology and Biophysics* (Eds. T. C. Ruch and H. D. Patton), p. 871, W. B. Saunders, Philadelphia (1965).
18. B. Deuticke and E. Gerlach, *Naunyn Schmiedebergs Arch. Pharmak. exp. Path.* **255,** 107 (1966).
19. V. F. W. Koss, G. Beisenherz and R. Maerkisch, *Arzneimittel-Forsch.* **12,** 1130 (1962).
20. R. D. Bunag, C. R. Douglas, S. Imai and R. M. Berne, *Circulation Res.* **15,** 83 (1964).
21. D. M. Aviado, H. Ito, Y. W. Cho and S. Bellet, *Cardialogia* **53,** 27 (1968).