Biochem. J. (1988) 249, 695–703 (Printed in Great Britain)

Absolute rates of adenosine formation during ischaemia in rat and pigeon hearts

Parviz MEGHJI, Kate M. MIDDLETON and Andrew C. NEWBY*
Department of Cardiology, University of Wales College of Medicine, Health Park, Cardiff CF4 4XN, Wales, U.K.

1. The activities of ecto- and cytosolic 5'-nucleotidase (EC 3.1.3.5), adenosine kinase (EC 2.7.1.20), adenosine deaminase (EC 3.5.4.4) and AMP deaminase (EC 3.5.4.6) were compared in ventricular myocardium from man, rats, rabbits, guinea pigs, pigeons and turtles. The most striking variation was in the activity of the ecto-5'-nucleotidase, which was 20 times less active in rabbit heart and 300 times less active in pigeon heart than in rat heart. The cytochemical distribution of ecto-5'-nucleotidase was also highly variable between species. 2. Adenosine formation was quantified in pigeon and rat ventricular myocardium in the presence of inhibitors of adenosine kinase and adenosine deaminase. 3. Both adenosine formation rates and the proportion of ATP catabolized to adenosine were greatest during the first 2 min of total ischaemia at 37 °C. Adenosine formation rates were 410±40 nmol/min per g wet wt. in pigeon hearts and 470±60 nmol/min per g wet wt. in rat hearts. Formation of adenosine accounted for 46% of ATP plus ADP broken down in pigeon hearts and 88% in rat hearts. 4. The data show that, in both pigeon and rat hearts, adenosine is the major catabolite of ATP in the early stages of normothermic myocardial ischaemia. The activity of ecto-5'-nucleotidase in pigeon ventricle (16±4 nmol/min per g wet wt.) was insufficient to account for adenosine formation, indicating the existence of an alternative catabolic pathway.

INTRODUCTION

Autoregulation of coronary blood flow in response to ischaemia is, at least in part, mediated by the vasodilator action of adenosine released after cleavage from cellular ATP (Berne, 1980). The ability of adenosine to decrease heart rate (James, 1965), to antagonize the inotropic effect of catecholamines both pre- and post-synaptically (Schrader *et al.*, 1977; Dobson, 1978, 1983; Hedqvist & Fredholm, 1979; Lokhandwala, 1979; Endoh & Yamashita, 1980) and increase atrioventricular conduction time (Drury & Szent-Gyorgi, 1929; Bellardinelli *et al.*, 1980, 1981) can also be seen as aspects of a compensatory response to limit ATP depletion (Newby, 1984). This beneficial role for adenosine may extend to other tissues (Newby, 1984). Nonetheless, when ischaemia is extreme or prolonged, nucleoside formation results in depletion of purine nucleotides, and this is associated with irreversible cellular injury (Hearse *et al.*, 1981). In the heart, in particular, recovery of purine nucleotide concentrations is delayed on reperfusion (De Boer *et al.*, 1980; Reimer *et al.*, 1981; Swain *et al.*, 1982*b*), owing to washout of nucleosides and bases (Swain *et al.*, 1982*b*) and to sluggishness of the '*de novo*' synthetic and salvage pathways (Swain *et al.*, 1982*a*; Ward *et al.*, 1984; Mauser *et al.*, 1985). Given the increasing practice of surgical cardioplegia and heart transplantation and the advent of thrombolytic therapy for the reversal of acute myocardial infarction, a deeper understanding of the pathways of nucleoside formation in ischaemic myocardium gains added importance.

This study was undertaken to determine the absolute rates of adenosine formation in myocardial tissue subjected to normothermic no-flow ischaemia. From such data it was possible to determine the proportions of AMP catabolized by deamination or dephosphorylation. The role of ecto-5'-nucleotidase was tested by comparing rates of adenosine formation in rat ventricles, in which the enzyme is abundant, and in pigeon ventricles, from which it is virtually absent.

MATERIALS AND METHODS

Chemicals

5-Iodotubercidin {4-amino-5-iodo-7-(β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine} was a gift from Dr. L. P. Townsend, College of Pharmacy, University of Michigan, Ann Arbor, MI, U.S.A., and EHNA [*erythro*-9-(2-hydroxy-3-nonyl)adenine] was a gift from Wellcome Research Laboratories, Beckenham, Kent, U.K. [2-^{3}H]-Adenosine (740–925 GBq/mmol) and [2-^{3}H]AMP (370–740 GBq/mmol) were obtained from Amersham International, Little Chalfont, Bucks., U.K. [2-^{3}H]IMP (200–250 GBq/mmol) was synthesized from [2-^{3}H]AMP as previously described (Worku & Newby, 1982). Anti-(rat liver ecto-5'-nucleotidase) serum was prepared in sheep as previously described (Stanley *et al.*, 1980; Meghji *et al.*, 1985).

Animals

Wistar rats (250–400 g), New Zealand White rabbits (2–4 kg) and Duncan Hartley guinea pigs (400–800 g) were obtained from colonies bred in the Animal Unit,

Abbreviations used: EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine; 5-iodotubercidin, 4-amino-5-iodo-7-(β-D-ribofuranosyl)pyrrolo[2,3-*d*]-pyrimidine.

* To whom correspondence should be addressed.

University of Wales College of Medicine. Domestic pigeons (*Columba livia*) were obtained from Abbot Brothers, Thuxton, Norfolk, U.K. European pond turtles (*Emys orbicularis*) were obtained from Biopet, Haverhill, Suffolk, U.K. Biopsies of human papillary muscle were obtained from nine patients [two male, seven female; average age 60 (range 42–70) years] undergoing a mitral-valve replacement operation. Rats, pigeons and turtles were killed by decapitation, and rabbits and guinea pigs were killed by cervical dislocation.

Enzymological studies

Hearts were removed and then exsanguinated by brief retrograde perfusion via the aorta with a solution of 154 mM-NaCl (except for turtles and man, where this was impracticable). Hearts were then cooled to 4 °C and part of ventricular tissue was weighed, dissected into cubes (approx. 4 mm × 4 mm × 4 mm) and homogenized in 9 vol. of buffer (20 mM-sodium dimethylglutarate, pH 7.0, 1 mM-EDTA, 0.1 mM-dithiothreitol). The tissue was homogenized at 4 °C with two 10 s bursts of a Polytronic homogenizer (Northern Media Supply, Hessle, N. Humberside, U.K.) at setting 2. Portions of each homogenate were centrifuged at 100000 *g* for 60 min at 4 °C, after which the soluble fraction was kept for analysis. Homogenates and soluble fractions were diluted appropriately with homogenizing buffer, before inclusion in the enzyme assays.

The ecto-5′-nucleotidase was measured by the method of Avruch & Wallach (1971) as modified by Newby *et al.* (1975). Incubations contained (final concns.) 50 mM-Tris/HCl, pH 8.0, 5 mM-$MgCl_2$, 0.2 mM-AMP (plus 1–2 kBq of [2-^{3}H]AMP, 1.5 mM-adenosine, 10 mM-sodium β-glycerophosphate, 0.1% Triton X-100 and homogenates at final dilutions of between 1:50 and 1:500. Reactions with each tissue were linear at 37 °C for 15 min. Non-specific AMPase was assayed in the same reaction mixture in which 0.5 mM-[$\alpha\beta$-methylene]ADP was substituted for 10 mM-β-glycerophosphate. In control studies 0.5 mM-[$\alpha\beta$-methylene]ADP inhibited rat liver 5′-nucleotidase by 96% under these conditions, whereas 10 mM-β-glycerophosphate inhibited rabbit intestine alkaline phosphatase (Sigma, type VIII) by 98%.

Soluble 5′-nucleotidase was measured as described by Worku & Newby (1983). The reaction mixture contained (final concns.) 70 mM-sodium β-glycerophosphate, pH 6.5, 5 mM-ATP, 35 mM-$MgCl_2$, 0.7 mM-mercaptoethanol, 2.1 mM-IMP (plus 500–600 Bq of [2-^{3}H]IMP) and soluble fraction at final dilution of 1:50 relative to the intact tissue. Reactions were linear at 37 °C for 40 min.

Adenosine kinase was measured under optimal conditions devised for rat hearts by Fisher & Newsholme (1984), by using a previously described ion-exchange filter method (De Jong & Kalkman, 1973; Newby, 1981). The reaction mixture contained (final concns.) 25 mM-sodium phosphate, pH 6.8, 2 mM-ATP, 1 mM-$MgSO_4$, 5 μM-adenosine (plus 15–20 kBq of [2-^{3}H]adenosine), 5 mM-phosphocreatine, 0.5 mg of creatine kinase/ml and soluble fraction at a final dilution of 1:1000 relative to the intact tissue. Assays were linear at 37 °C for all tissues up to 10 min.

Adenosine deaminase was measured at 37 °C by the decrease in A_{265} (Kalckar, 1947) in a reaction mixture containing 50 mM-sodium phosphate, pH 7.0, 50 μM-adenosine and soluble fraction at a final dilution of 1:600 relative to intact tissue.

AMP deaminase was measured under optimal conditions determined by Moss (1977). The reaction mixture (0.5 ml) contained 20 mM-sodium dimethylglutarate, pH 7.0, 1 mM-EDTA, 0.1 mM-dithiothreitol, 150 mM-KCl, 1 μM-EHNA (to inhibit adenosine deaminase) and 25 mM-AMP as substrate. Reactions were initiated by adding 10 μl of soluble fractions at 1:10 dilution relative to the intact tissue. Reactions were linear at 37 °C for 20 min. Blank incubations were conducted in which AMP was omitted. Reactions were terminated with 0.05 ml of 50% (w/v) $HClO_4$. After centrifugation at 1500 *g* for 5 min, 0.5 ml of supernatant was taken and neutralized at 4 °C with 0.125 ml of a solution of 1.2 M-K_2CO_3/30 mM-EDTA. After centrifugation at 1500 *g* for 5 min at 4 °C, supernatants (0.5 ml) were taken and the concentration of NH_3 present in them was determined by the method of Chaney & Marbach (1962).

Cytochemical location of ecto-5′-nucleotidase

Precipitates of lead phosphate produced after hydrolysis of AMP in the presence of $Pb(NO_3)_2$ were located after conversion into PbS (Wachstein & Miesel, 1957). Briefly, cryotome sections (10–15 μm) were obtained from unfixed ventricular tissue maintained at −20 °C. Sections were air-dried on chrome-gel-coated slides at 23 °C for 1 h and then fixed for 2 min with the fixative of Karlsson & Schultz (1965). Lead phosphate was generated by incubation of fixed sections at 23 °C for 30–60 min in a solution containing 1 mM-AMP, 0.25 M-sucrose, 2 mM-$Pb(NO_3)_2$, 5 mM-$Mn(NO_3)_2$ and 50 mM-Tris/HCl, pH 6.8. AMP was omitted from control incubations. After rinsing with distilled water, sections were incubated for 1 min at 23 °C with an aqueous solution of ammonium polysulphide (BDH, Poole, Dorset, U.K.) equivalent to a final concn. of approx. 1% (w/v) H_2S. Slides were rinsed with water, dried and mounted with Canada Balsam.

Measurement of purine catabolism during myocardial ischaemia

Rat or pigeon hearts were removed and perfused at 37 °C via the aorta for approx. 5 min, at 20 ml/min, with Krebs–Ringer bicarbonate buffer containing 1 mM-pyruvate equilibrated with O_2/CO_2 (19:1). Hearts were then perfused at 23 °C over a 30 s period with 10 ml of a solution of 154 mM-NaCl, 1 μM-5-iodotubercidin and 10 μM-EHNA. In experiments to determine recovery of adenosine, the solution also contained 0.5 mM-adenosine plus 200 kBq of [2-^{3}H]adenosine/ml. Hearts were then removed from the aortic cannula, and the ventricles were divided with a scalpel into four approximately equal portions. One portion was immediately frozen between tongs cooled in liquid N_2. The other three portions were placed in tared stoppered tubes containing 1 ml of the final perfusing solution (lacking added adenosine in all experiments). The tubes were then reweighed and incubated at 37 °C. After 2, 5 and 20 min one tube was opened, the ventricular tissue was removed, blotted and then freeze-clamped while trichloroacetic acid [0.1 ml of a 50% (w/v) solution] was added to the residual solution.

Purine metabolite concentrations were measured by h.p.l.c. methods in $HClO_4$ extracts of tissues and neutralized residual solutions as described previously (Newby *et al.*, 1983). Recoveries of adenosine were

established by collecting the eluate containing adenosine from the h.p.l.c. column. The radioactivity in the eluate was then compared with the total radioactivity present in the samples before h.p.l.c.

ATP, phosphocreatine, creatine and P_i concentrations were measured in tissue extracts immediately after neutralization, by using standard u.v.-spectroscopic methods (Bergmeyer, 1974; Wahler & Wollenberger, 1958). The total contents of metabolites in the tissue extracts and residual solutions were summed and expressed relative to the wet weight of the sample of ventricular tissue.

Statistical methods

Values are expressed throughout as means ± S.E.M. Sets of data were compared by Student's *t* test for unpaired data.

RESULTS

Activities of adenosine-forming and -metabolizing enzymes and of AMP deaminase in hearts of different species

Striking differences were observed in the activity and cytochemical distribution of ecto-5′-nucleotidase in the hearts of the species studied (Table 1, column 1; Fig. 1). The enzyme was abundant in ventricular muscle of rats and guinea pigs (Table 1), but in rats there was markedly less activity associated with arterial smooth muscle (cf. Figs. 1*a* and 1*b*). In human papillary muscle and turtle ventricle, the lower total activity (Table 1) was distributed on both cardiac and smooth-muscle myocytes (Figs. 1*c* and 1*f*). The activity in rabbit ventricle was 20-fold less than in rat ventricle (Table 1), and appeared almost confined to arterial smooth muscle (Fig. 1*d*). In pigeon ventricle, the ecto-5′-nucleotidase was almost absent (Table 1, Fig. 1*f*). In rat, guinea-pig and rabbit ventricle, where this was investigated, a proportion of the homogenate activity measured under conditions optimal for ecto-5′-nucleotidase was recovered in the soluble fraction (Table 1, column 3). The activity was greatly inhibited by 1:100 dilution of anti-(ecto-5′-nucleotidase) serum to 9±3 (6) nmol/min per g wet wt. in rat, to 4±2 (8) nmol/min per g wet wt. in guinea-pig and to 4±1 (4) nmol/min per g wet wt. in rabbit ventricle. In separate studies this dilution of the same antiserum was shown to inhibit homogenate 5′-nucleotidase by 98.7±0.3 (10)% in rat, 96±4 (4)% in guinea-pig and 98±1 (5)% in rabbit ventricle.

Non-specific phosphatase activity was also observed in myocardial homogenates of all species, and was also greatest in rats and least in pigeons (Table 1, column 2), although it did not vary in fixed proportion to the ecto-5′-nucleotidase activity.

A distinct 5′-nucleotidase was observed in the soluble fraction in the presence of ATP. The activity was measured in the presence of β-glycerophosphate to inhibit the non-specific phosphatases and was not decreased by either anti-(ecto-5′-nucleotidase) serum or [αβ-methylene]ADP (results not shown). The enzyme was slightly more active in pigeon than rat ventricle and 3–4-fold less active in turtle and human.

Adenosine kinase was present in ventricular myocardium of all the species studied, with only a 4-fold difference between the most active (rat) and least active (rabbit) hearts (Table 1). Adenosine deaminase was also present in all hearts studied, but was less active in rabbit and turtle ventricle than in other species (Table 1). AMP deaminase was, by contrast, most active in turtle ventricle, whereas guinea-pig and human ventricular myocardium had the lowest activities (Table 1).

Ischaemia-induced nucleotide breakdown and adenosine formation in pigeon ventricular myocardium

Pigeon ventricular myocardium suffered a 60% decrease in ATP concentration after 5 min of normothermic no-flow ischaemia, and an 88% decrease after 20 min (Table 2). Phosphocreatine became exhausted by 5 min, whereas the ADP concentration was decreased by only 47% at 20 min. The concentrations of AMP and IMP increased 6-fold and 16-fold, respectively, over 20 min (Table 2). Inosine accumulated as the principal catabolite of ATP, accounting for 71% of the fall in ATP and ADP concentrations at 5 min and 77% at 20 min. There was no loss of total purines. Adenosine accumulated transiently at an apparent rate of 27 nmol/min per g wet wt., to account for 5.1% of the fall in ATP plus ADP concentrations at 5 min (Table 2).

Table 1. Activities of adenosine-forming and -metabolizing enzymes and of AMP deaminase in hearts

Enzyme activities in homogenates or soluble fractions (100000 *g*, 60 min supernatants) of samples of ventricular myocardium from six to ten hearts (four to six in man) as described in the Materials and methods section. Values are means ± S.E.M.; N.D., not done.

	Enzyme activity (nmol/min per g wet wt.)						
	Homogenate		Soluble fraction				
Species	Ecto-5′-nucleotidase	Non-specific phosphatase	Ecto-5′-nucleotidase	ATP-activated 5′-nucleotidase	AMP deaminase	Adenosine kinase	Adenosine deaminase
Rat	4900±300	900±100	400±100	410±40	3700±300	80±7	640±40
Guinea pig	3500±400	680±40	470±50	340±30	900±100	36±6	830±30
Man	560±50	140±40	N.D.	150±20	720±80	33±9	410±70
Turtle	510±50	165±8	N.D.	116±8	9000±400	44±4	68±8
Rabbit	250±30	82±8	60±10	270±30	2200±400	24±4	190±20
Pigeon	16±4	34±5	N.D.	590±40	2000±400	34±4	650±30

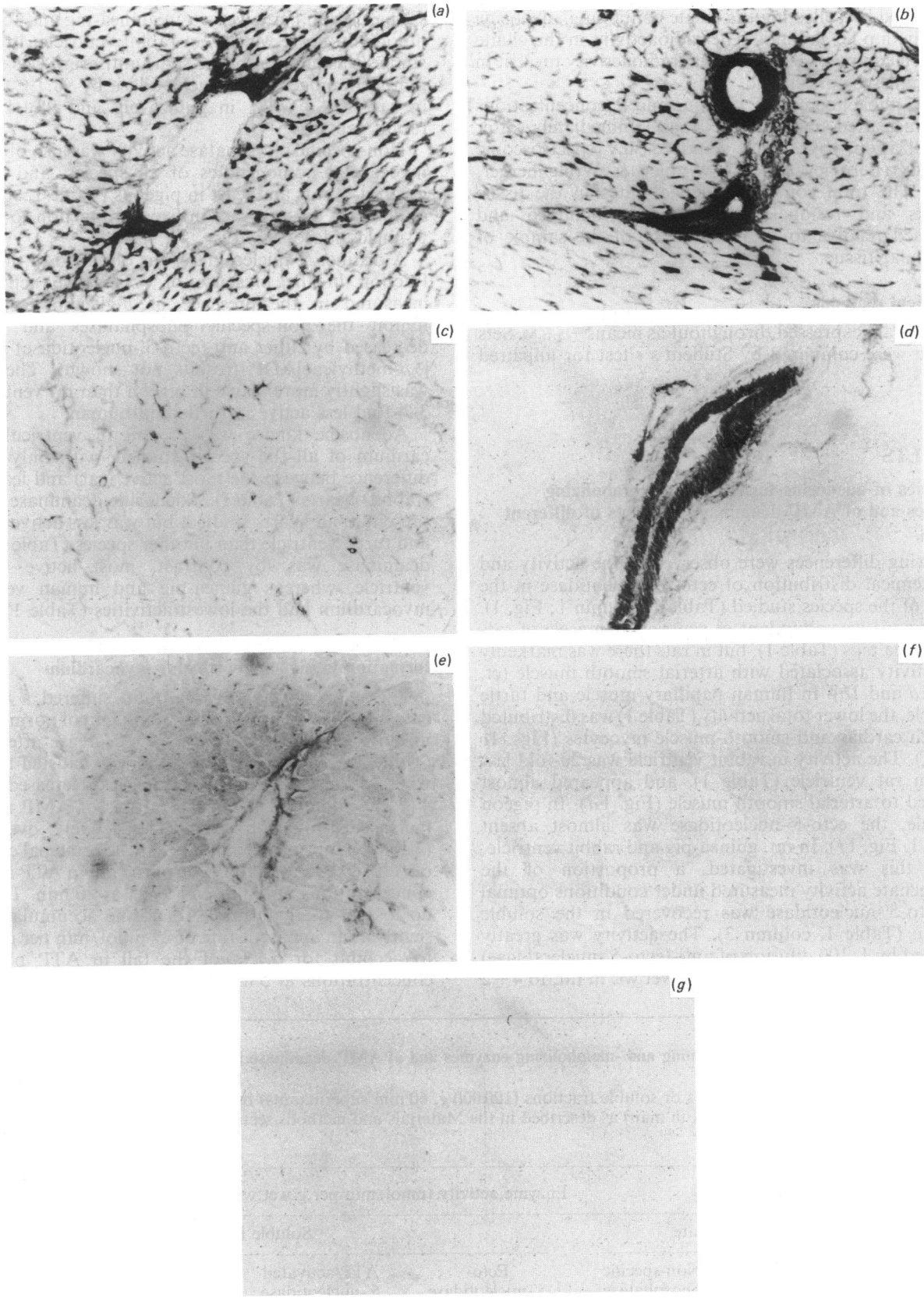

Fig. 1. Cytochemical distribution of ecto-5′-nucleotidase

Frozen sections of rat (*a*), guinea-pig (*b*), turtle (*c*), rabbit (*d*), human (*e*) and pigeon (*f*) ventricular myocardium were incubated with AMP in the presence of $Pb(NO_3)_2$. Lead phosphate precipitates were then observed, after conversion into PbS, by light microscopy. Panel (*g*) shows the staining obtained with rat myocardium when AMP was omitted. Magnification approx. × 50.

Table 2. Effect on pigeon ventricle metabolites of ischaemia

Metabolite concentrations were determined in portions of hearts either by h.p.l.c. methods or by u.v. spectrometry. Hearts were made ischaemic for the times shown. Values are means ± S.E.M.

	Metabolite concn. (nmol/g wet wt.)		
Time (min) ...	0	5	20
ATP (by h.p.l.c.)	4200 ± 400	1700 ± 300	500 ± 200
ATP (by spectrometry)	3900 ± 400	1500 ± 300	300 ± 200
ADP	1500 ± 100	1330 ± 80	800 ± 200
AMP	180 ± 20	630 ± 80	1100 ± 100
IMP	10 ± 10	110 ± 10	160 ± 20
Adenosine	44 ± 7	180 ± 40	54 ± 8
Inosine	400 ± 70	2300 ± 200	3800 ± 200
Hypoxanthine	80 ± 10	80 ± 10	110 ± 10
Total purines (by h.p.l.c.)	6300 ± 500	6300 ± 200	6500 ± 300
Phosphocreatine	4100 ± 500	0	0
Creatine	12000 ± 800	9600 ± 400	12000 ± 2000
P_i	10200 ± 1200	19000 ± 1000	24000 ± 2000

Table 3. Effect on pigeon ventricle metabolite concentrations of ischaemia in the presence of 5-iodotubercidin and EHNA

Metabolite concentrations were determined in portions of six hearts either by h.p.l.c. methods or by u.v. spectrometry. Hearts were made ischaemic for the times shown. Values are means ± S.E.M.

	Metabolite concn. (nmol/g wet wt.)			
Time (min) ...	0	2	5	20
ATP (by h.p.l.c.)	3300 ± 300	1400 ± 200	600 ± 100	110 ± 40
ATP (by spectrometry)	3500 ± 300	1700 ± 200	900 ± 100	110 ± 60
ADP	1170 ± 70	1300 ± 100	1000 ± 100	470 ± 70
AMP	170 ± 20	490 ± 60	660 ± 90	1030 ± 60
IMP	40 ± 10	100 ± 10	120 ± 20	190 ± 10
Adenosine	120 ± 30	940 ± 110	890 ± 150	360 ± 30
Inosine	930 ± 140	1400 ± 200	2100 ± 200	4700 ± 300
Hypoxanthine	47 ± 7	61 ± 7	51 ± 4	80 ± 10
Total purines (by h.p.l.c.)	5800 ± 300	5800 ± 300	5400 ± 400	6900 ± 300
Phosphocreatine	5800 ± 1300	600 ± 400	200 ± 200	400 ± 400
Creatine	12000 ± 1000	14000 ± 2000	11300 ± 400	16000 ± 1000
P_i	10000 ± 2000	17000 ± 1000	18000 ± 2000	25000 ± 2000

In the presence of 5-iodotubercidin, an inhibitor of adenosine kinase (Wotring & Townsend, 1979), and EHNA, and inhibitor of adenosine deaminase (Agarwal *et al.*, 1977), ischaemia resulted in a significantly ($P < 0.05$) greater decrease in ATP concentration at 5 min (82%) and at 20 min (97%) (Table 3). The decrease in ADP concentration at 20 min and the increases in AMP and IMP concentrations were similar to those observed during ischaemia in the absence of the drugs. Inosine remained the major catabolite of ATP at 20 min, accounting for 97% of the fall in ATP plus ADP concentration. At 5 min, however, it accounted for only 41%, and at 2 min only 27%, of ATP and ADP breakdown. Adenosine accumulated rapidly during the first 2 min of ischaemia (Table 3); its concentration remained elevated at 5 min but was lower by 20 min. Adenosine concentration was significantly ($P < 0.001$) greater at both 5 min and 20 min of ischaemia than in the absence of 5-iodotubercidin and EHNA.

In a separate series of hearts, the effectiveness of 5-iodotubercidin and EHNA in preventing adenosine breakdown was assessed by co-infusing [^{3}H]adenosine at the average concentration between 0 and 2 min of ischaemia. Adenosine metabolism was assessed separately in the tissue pieces and in the medium in which they were incubated. Substantial adenosine metabolism occurred before the 0 min time point, but subsequent losses amounted to less than 25% in the tissue pieces and 5% in the suspending medium at the 2 and 5 min time points (Fig. 2). At the 20 min time point, 74% of the adenosine present at 0 min had been metabolized in the tissue pieces and 25% in the suspending medium (Fig. 2). The data were used to calculate approximate first-order rate constants for adenosine removal, and these were used, in turn, to calculate the effect of adenosine metabolism on estimates of adenosine formation. As shown in Fig. 3, metabolism resulted in only a minor underestimation of adenosine formation at the 2 min and 5 min time points, but appeared to account for the loss of adenosine between 5 and 20 min. From this

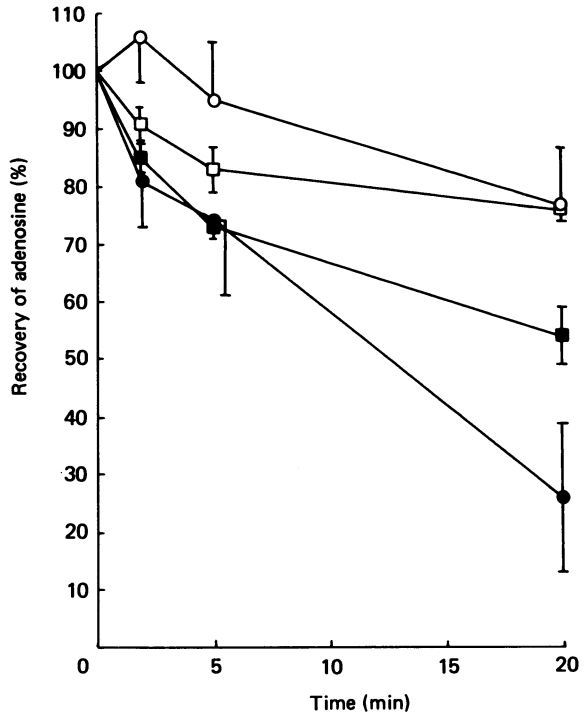

Fig. 2. Recovery of adenosine infused into hearts in the presence of 5-iodotubercidin and EHNA

[^{3}H]Adenosine (0.5 mM) was infused into pigeon or rat hearts ($n = 6$) at 23 °C together with a perfusion medium consisting of 154 mM-NaCl, 1 μM-5′-iodotubercidin and 10 μM-EHNA. A segment of the ventricular tissue was then freeze-clamped and extracted with $HClO_4$. The recovery of [^{3}H]adenosine was 58±8% in pigeon and 91±2% in rat hearts. Further segments were incubated at 37 °C for the times shown in 1 ml of perfusion medium, after which tissue segments and suspending medium were separately extracted. The recovery of [^{3}H]adenosine in pigeon tissue (●), pigeon perfusion medium (○), rat tissue (■) and rat perfusion medium (□) was then determined and expressed as a percentage of the recovery from unincubated tissue. Values are means±S.E.M. The data were used to calculate first-order rate constants for adenosine metabolism in pigeon tissue (0.067 min^{-1}), pigeon perfusion medium (0.014 min^{-1}), rat tissue (0.028 min^{-1}) and rat perfusion medium (0.012 min^{-1}).

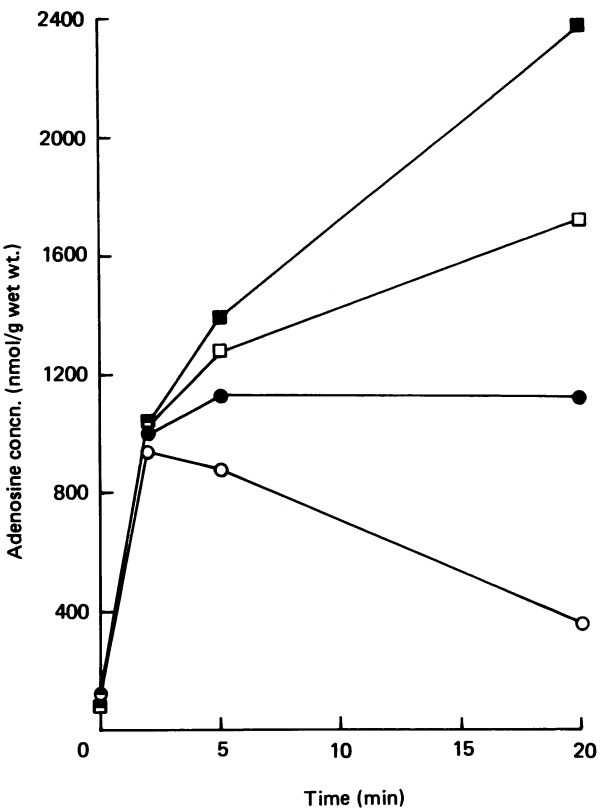

Fig. 3. Measured and corrected estimates of adenosine formation

The mean total concentration of adenosine in tissue and incubation medium was replotted for pigeon hearts (○) from Table 3 and for rat hearts (□) from Table 4. Adenosine metabolism (M_n) between times t_n and t_{n-1} was estimated by using first-order rate constants (k) derived from the data in Fig. 2 and the approximation

$$M_n = k(t_n - t_{n-1})(A_n + A_{n-1})/2$$

where A_n was the measured adenosine concentration in tissue or incubation medium at t_n. Corrected estimates of adenosine formed (C_n) in pigeon (●) and rat (□) hearts were then calculated from the equation

$$C_n = (A_n + M_n)_{\text{tissue}} + (A_n + M_n)_{\text{incubation medium}}.$$

analysis, it is clear that the apparent slowing of adenosine formation after 2 min of ischaemia cannot be explained by adenosine metabolism.

Adenosine accumulation accounted for 46% of the fall in ATP and ADP concentration at 2 min, 27% at 5 min and 6.2% at 20 min. After correcting for adenosine metabolism these values were 50%, 35% and 26% respectively. The rate of adenosine formation over the first 2 min of ischaemia was 410±40 nmol/min per g wet wt., or 450 nmol/min per g wet wt. after correction for metabolism.

Ischaemia-induced nucleotide breakdown and adenosine formation in rat ventricular myocardium

Ischaemia in the presence of 5-iodotubercidin and EHNA caused a similar pattern of changes in purine metabolites in rat and pigeon ventricles (cf. Tables 3 and 4). ATP concentration was decreased by 94% at 20 min, whereas phosphocreatine was exhausted by 5 min. ADP concentration rose transiently and then declined, whereas both AMP and IMP concentrations rose monotonically (Table 4). Inosine accumulated to account for 34%, 25% and 21% of ATP and ADP degradation at 2, 5 and 20 min of ischaemia, whereas hypoxanthine accounted for 11%, 6% and 9% respectively (Table 4). Adenosine was, however, the major catabolite of ATP and ADP at all times. Its accumulation accounted for 88%, 55% and 43% of the fall in ATP and ADP concentration at 2, 5 and 20 min respectively.

Metabolism of adenosine in the presence of 5-iodotubercidin and EHNA was less pronounced in rat than in pigeon ventricle (Fig. 2), and the effect of adenosine metabolism on estimates of adenosine formation was therefore correspondingly less (Fig. 3). After correction for adenosine metabolism, 92%, 59% and 56% of the fall in ATP and ADP concentrations was accounted for by adenosine formation.

Table 4. Effect on rat ventricle metabolite concentrations of ischaemia in the presence of 5-iodotubercidin and EHNA

	Metabolite concn. (nmol/g wet wt.)			
Time (min) ...	0	2	5	20
ATP (by h.p.l.c.)	3700±300	2200±200	1300±200	230±40
ATP (by spectrometry)	3300±100	1800±200	1100±200	190±50
ADP	700±100	1150±50	920±80	340±30
AMP	290±50	600±100	600±100	1200±60
IMP	43±2	300±50	250±40	340±90
Adenosine	80±10	1000±100	1280±60	1720±90
Inosine	35±4	390±20	580±20	840±40
Hypoxanthine	60±10	180±30	190±20	390±50
Total purines (by h.p.l.c.)	4900±300	5800±200	5200±300	5100±300
Phosphocreatine	4400±300	400±200	100±100	30±100
Creatine	6000±1000	11000±2000	11000±1000	9000±2000
P_i	2400±700	12000±2000	13000±3000	16000±2000

The rate of adenosine formation was, as in pigeon ventricle, greatest in the first 2 min of ischaemia (Fig. 3), although it declined less steeply. Adenosine formation proceeded at 470±60 nmol/min per g wet wt. over the first 2 min of ischaemia, or 480 nmol/min per g wet wt. after correction for metabolism.

DISCUSSION

The distribution of adenosine-forming and -metabolizing enzymes and of AMP deaminase are in broad agreement, in areas of overlap, with previous studies (Nakatsu & Drummond, 1972; Arch & Newsholme, 1978). We confirmed, for example, that AMP deaminase activity was high in turtle ventricle (Nakatsu & Drummond, 1972) and that ecto-5′-nucleotidase activity was low in pigeon ventricle (Nakatsu & Drummond, 1972; Arch & Newsholme, 1978). We did not confirm a similarly low 5′-nucleotidase activity in turtle ventricle, although the species of turtle used by Nakatsu & Drummond (1972) was not stated. 5′-Nucleotidase activity measured with AMP as substrate and in the absence of ATP has been previously described in post-microsomal supernatant fractions of guinea-pig hearts (Schutz *et al.*, 1981). Our results show that this and similar activities in soluble fractions of rat and rabbit hearts result from an enzyme identifiable immunologically as the plasma-membrane ecto-5′-nucleotidase, in agreement with Fritzon *et al.* (1986), who studied rat liver. The provenance of the enzyme is uncertain, but it is possible that it derives from the plasma membrane during homogenization (Fritzon *et al.*, 1986). Evidence has been presented that the enzyme is attached to the plasma membrane by a phospholipid tail (Low, 1987). If so, solubilization could have occurred as a result of phospholipase activation.

The most novel finding of our cytochemical study was the concentration of ecto-5′-nucleotidase activity on to vascular smooth muscle in the rabbit ventricle. It is unlikely that this resulted from selective inactivation of a pool of enzyme, since the overall staining intensity varied in the same order (rat and guinea pig > human and turtle > rabbit > pigeon) as the measured homogenate activity. Moreover, the location of the enzyme observed in rat, human and guinea-pig tissue was similar to that reported by Nakatsu & Drummond (1972), who also demonstrated location on vascular myocytes in guinea-pig, but not rat, ventricle. Possible location of 5′-nucleotidase on endothelial cells was not investigated, owing to the low resolving power of light microscopy. A highly variable species distribution of ecto-5′-nucleotidase has also been reported in the hippocampus (Lee *et al.*, 1986).

Non-specific AMPase activity was also present in homogenates from all hearts studied. In most species its activity was 3–10-fold less than ecto-5′-nucleotidase, but in the pigeon it was 2-fold greater.

The cytosolic 5′-nucleotidase was measured with ATP present both to activate the enzyme (Van den Berghe *et al.*, 1977) and to inhibit the ecto-enzyme (Burger & Lowenstein, 1975). Since the purified soluble 5′-nucleotidase has approximately the same $V_{max.}$ for AMP and IMP (Itoh & Oka, 1985; Itoh *et al.*, 1986), and since AMP is metabolized in competing reactions by AMP deaminase and adenylate kinase, IMP was used as substrate. Soluble 5′-nucleotidase was present in hearts of all species examined, including man.

Ischaemia-induced adenosine formation

The protocol for inducing ischaemia used by Gerlach and co-workers (Gerlach *et al.*, 1963; Deuticke & Gerlach, 1966) was chosen for three main reasons. First and foremost, the inhibitor, 5-iodotubercidin, is so scarce that it could not be used in a flow-through system. Secondly, our intention was to produce the most rapid possible ATP degradation, so as to approach the maximum rate at which the heart could generate adenosine. Thirdly, it was a convenient model in which to account for all the metabolites of ATP, and hence to assess their relative quantitative importance. The relevance of this model to the transient mild ischaemia which presumably precedes an autoregulatory response is more questionable. It is interesting, however, that the most rapid adenosine formation took place in the first minutes of ischaemia, when it is unlikely that any gross ischaemic changes such as structural disruption had taken place (Hearse *et al.*, 1981).

The rates of adenosine formation were grossly underestimated in the absence of inhibitors of adenosine metabolism. This may explain, in part, why the rates of adenosine formation measured here were 10–100 times those obtained in most previous studies (Gerlach *et al.*,

1963; Richman & Wyborny, 1964; Deuticke & Gerlach, 1966; Rubio *et al.*, 1973; Degenring *et al.*, 1975; Frick & Lowenstein, 1976; Schrader & Gerlach, 1976; Foley *et al.*, 1978; Schutz *et al.*, 1981; Edlund *et al.*, 1983). Studies with ischaemic rabbit heart, which we showed to contain low activities of adenosine kinase and adenosine deaminase, gave rates of adenosine production similar to those obtained here (Imai *et al.*, 1964). Differences in myocardial adenosine concentrations between systole and diastole in isolated guinea-pig hearts (Thompson *et al.*, 1980) also suggest rates of adenosine formation of this higher order.

The rate of adenosine formation in ischaemic pigeon hearts was 25 times the activity of ecto-5'-nucleotidase in this tissue, and 12 times the activity of the non-specific AMPase. This provides further clear evidence for an alternative pathway for myocardial adenosine production, which does not involve the ecto-5'-nucleotidase (cf. Frick & Lowenstein, 1976; Schutz *et al.*, 1981; Meghji *et al.*, 1985). Pigeon hearts contained a soluble 5'-nucleotidase, and there is preliminary evidence (Newby & Meghji, 1986) that the enzyme shows sufficient activity with AMP as substrate to explain ischaemia-induced adenosine formation.

The rate of adenosine formation in ischaemic rat hearts was more than 70 times the activity of *S*-adenosylhomocysteine hydrolase reported for the same tissue (Schrader *et al.*, 1981), indicating that the transmethylation pathway contributed little to ischaemia-induced adenosine formation. There was more than sufficient ecto-5'-nucleotidase and just sufficient soluble 5'-nucleotidase or non-specific AMPase to explain adenosine formation, provided that the enzymes are active towards AMP under the conditions of the cell. The exclusively extra-cytoplasmic location of the ecto-5'-nucleotidase (Stanley *et al.*, 1980), and its almost total inhibition by cytosolic concentrations of ATP (Burger & Lowenstein, 1975), argue against its role in hydrolysis of cytoplasmic AMP. The K_m for AMP of the purified cytosolic 5'-nucleotidase (2.6–10 mM; Worku & Newby, 1983; Itoh & Oka, 1985; Itoh *et al.*, 1986) and its measured activity suggest, however, that it may account for only part of the rate of adenosine formation measured here, given the AMP concentrations (1–2 mM) achieved in ischaemic hearts. The possible contribution of the non-specific phosphatase needs to be investigated further.

Adenosine was the major catabolite of ATP during ischaemia in both pigeon and rat hearts. This confirms the work of Achterberg *et al.* (1985), using low-flow ischaemia in rat hearts perfused with EHNA alone. Since the combination of 5-iodotubercidin and EHNA had no influence on the concentrations of AMP or IMP present during ischaemia, there was no evidence that the agents inhibited AMP deaminase and hence favoured dephosphorylation of AMP. The observation that 5-iodotubercidin and EHNA accelerated ATP breakdown in pigeon hearts may be explained by inhibition of reconversion of adenosine to AMP. The proportion of ATP breaking down to adenosine in pigeon hearts (46%) or rat hearts (88%) was much greater than values of 18% in Ehrlich ascites-tumour cells (Lomax & Henderson, 1973) or cultured neonatal heart cells (Meghji *et al.*, 1985) and 6% in polymorphonuclear leucocytes (Newby & Holmquist, 1981), although in all cases adenosine formation was greatest during the early phase of ATP degradation. The propensity of the heart for adenosine formation is interesting in view of its putative regulatory roles.

The strategy presented here for the measurement of absolute rates of adenosine formation is readily applicable to other tissues. These rate measurements are important, since any proposed pathway for adenosine formation must account for both the rate and time course of adenosine formation (Worku & Newby, 1983). The ability of particular organs and particular cell types within organs to maintain high rates of adenosine formation will, moreover, be useful in evaluating the importance of adenosine-mediated regulation in those tissues and in elucidating the sites of adenosine production.

We thank Professor G. W. Kreutzberg for advice and instruction on the cytochemical location of 5'-nucleotidase. The work was supported by a grant from the Medical Research Council.

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Received 13 July 1987/7 September 1987; accepted 29 September 1987