

# The transmethylation pathway as a source for adenosine in the isolated guinea-pig heart

Hilary G. E. LLOYD, Andreas DEUSSEN, Hartwig WUPPERMANN and Jürgen SCHRADER\*

Physiologisches Institut I, Universität Düsseldorf, Moorenstrasse 5, 4000 Düsseldorf 1, Federal Republic of Germany

In order to quantify adenosine production from the transmethylation pathway [*S*-adenosylmethionine (AdoMet) → *S*-adenosylhomocysteine (AdoHcy) ⇌ adenosine + L-homocysteine] in the isolated guinea-pig heart under basal conditions (normoxic perfusion with 95% O<sub>2</sub>) and during elevated adenosine production (hypoxic perfusion with 30% O<sub>2</sub>), two methods were used. (1) Hearts were perfused with normoxic medium containing [2,5,8-<sup>3</sup>H]adenosine (5 μM) and L-homocysteine thiolactone (0.1 mM), which brings about net AdoHcy synthesis via reversal of the AdoHcy hydrolase reaction and labels the intracellular pool of AdoHcy. From the decrease in AdoHcy pool size and specific radioactivity of AdoHcy in the post-labelling period, the rate of transmethylation, which is equivalent to the rate of adenosine production, was calculated to be 0.98 nmol/min per g. Adenosine release from the hearts was 40–50 pmol/min per g. (2) Hearts were perfused with hypoxic medium containing [<sup>35</sup>S]homocysteine (50 μM). Owing to the hypoxia-induced increase in adenosine production, this procedure also results in expansion and labelling of the AdoHcy pool. From the dilution of the specific radioactivity of AdoHcy relative to that of [<sup>35</sup>S]homocysteine, the rate of AdoHcy synthesis from AdoMet (transmethylation) was calculated to be 1.12 nmol/min per g. It is concluded that in the oxygenated heart the transmethylation pathway is quantitatively an important intracellular source of adenosine, which exceeds the rate of adenosine wash-out by the coronary system by about 15-fold. Most of the adenosine formed by this pathway is re-incorporated into the ATP pool, most likely by adenosine kinase. The transmethylation pathway is essentially O<sub>2</sub>-independent, and the known hypoxia-induced production of adenosine must be derived from an increase in 5'-AMP hydrolysis.

## INTRODUCTION

The actions of adenosine to cause coronary vasodilation, slowing of atrio-ventricular conduction and decreased sinus rate were first described by Drury & Szent-Györgyi (1929). Subsequently it was proposed that adenosine may be an important regulator of coronary blood flow because of the close correlation between adenosine production and myocardial energy metabolism (Berne, 1963; Gerlach *et al.*, 1963). More recent evidence has supported the original suggestion by Berne (1963) that the major stimulus for myocardial adenosine formation is a decrease in tissue oxygenation which results from an imbalance between oxygen supply and demand (Bardenheuer & Schrader, 1986). This suggests that the metabolic route by which adenosine is formed by the heart must be closely linked to tissue oxygenation.

Adenosine production arises from ATP degradation via 5'-AMP hydrolysis. The key enzyme responsible for adenosine formation from 5'-AMP is 5'-nucleotidase, which in the heart is found largely as an ecto-enzyme (Olsson *et al.*, 1973; Baer & Drummond, 1968). Owing to the predominantly extracellular location of 5'-nucleotidase, it has been assumed that much of the adenosine is formed in the extracellular compartment of the heart (Rubio *et al.*, 1973; see Berne, 1980). More recently a cytosolic 5'-nucleotidase has been described (Lowenstein *et al.*, 1983; Schrader, 1983); therefore, adenosine may also be formed intracellularly and reach the extracellular space by facilitated diffusion along its concentration

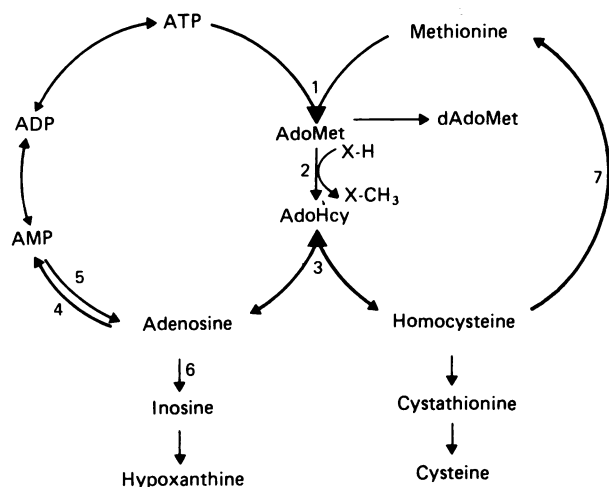
gradient. The first support for the intracellular formation of adenosine was obtained from the experiments of Schütz *et al.* (1981), in which it was demonstrated that inhibition of the ecto-5'-nucleotidase by adenosine 5'-[αβ-methylene]diphosphate had no effect on the hypoxia-induced release of adenosine from the isolated guinea-pig heart. By a similar approach, intracellular adenosine formation has been demonstrated in neonatal-rat heart cells in culture (Meghji *et al.*, 1985).

In addition to ATP degradation via 5'-AMP hydrolysis, adenosine can be formed from the transmethylation pathway, which involves the transfer of the methyl group of AdoMet to a variety of methyl acceptors (see Fig. 1). AdoMet was discovered and identified by Cantoni (1953) and is synthesized from methionine and ATP by the reaction catalysed by the adenosyltransferase. After methyl transfer AdoMet forms AdoHcy, the latter subsequently being hydrolysed by AdoHcy hydrolase to adenosine and L-homocysteine. This reaction is reversible, with the equilibrium lying far in the direction of AdoHcy synthesis (equilibrium constant 10<sup>-6</sup> M). Normally, however, the reaction proceeds in the direction of hydrolysis, because both reaction products are metabolized further, adenosine by adenosine kinase or adenosine deaminase and homocysteine by methionine synthase or cystathionine β-synthase (De la Haba & Cantoni, 1959).

AdoHcy hydrolase in cardiac tissue is an exclusively cytosolic enzyme (Schütz *et al.*, 1981) and has been found to have substantial activity in the hearts of several

Abbreviations used: AdoHcy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine; EHNA, erythro-9-(2-hydroxynon-3-yl)adenine.

\* To whom reprint requests should be addressed.



**Fig. 1. Pathway of adenosine and homocysteine production from the transmethylation pathway**

Abbreviations: dAdoMet = deoxyAdoMet; X = methyl acceptor. Enzymes: 1, ATP:L-methionine adenosyltransferase (EC 2.5.1.6); 2, S-adenosyl-L-methionine:X methyltransferase (EC 2.1.1.); 3, S-adenosyl-L-homocysteine hydrolase (EC 3.3.1.1); 4, adenosine kinase (EC 2.7.1.20); 5, 5'-nucleotidase (EC 3.1.3.5); 6, adenosine deaminase (EC 3.5.4.4); 7, 5'-methyltetrahydrofolate-homocysteine methyltransferase (EC 2.1.1.13).

species, including human (Schrader, 1983). The transmethylation pathway therefore may be an intracellular source of adenosine, in addition to ATP catabolism via 5'-AMP. Previous work has indicated that the transmethylation pathway could be contributing as much as 0.6 nmol/min per g to adenosine production in the isolated guinea-pig heart, which is more than 10 times higher than the basal release of adenosine from this preparation measured in the venous effluent during normoxic perfusion (Schrader, 1983).

The aim of the present study was to measure, in the isolated guinea-pig heart, the overall rate of cellular transmethylation, which, under steady-state conditions, can be taken to be equivalent to the rate of formation of adenosine by this pathway. In order to establish whether the flux rate through this pathway is altered at a time when cardiac adenosine production is markedly increased, transmethylation was measured under both normoxic and hypoxic perfusion conditions.

## EXPERIMENTAL

### Materials

[2,5',8-<sup>3</sup>H]Adenosine (40–50 Ci/mmol) was purchased from Amersham Buchler. [L-<sup>35</sup>S]Homocysteine (6–8 mCi/mmol) was synthesized from [L-<sup>35</sup>S]methionine (> 800 Ci/mmol; Amersham Buchler) by Dr. K. Hamacher, Department of Nuclear Chemistry, Kernforschungsanlage Jülich. erythro-9-(2-Hydroxynon-3-yl)-adenine (EHNA) was a gift from Burroughs Wellcome. L-Homocysteine thiolactone was purchased from Sigma. NOVA-PAK C<sub>18</sub> (5 μm; dimensions 8 mm × 10 cm) (Radial-PAK) was purchased from Waters Associates.

### Animal experiments

Hearts from guinea pigs (200–350 g) were rapidly excised and perfused via the aorta by the Langendorff technique. An aortic pressure of 60 cmH<sub>2</sub>O was used, and the perfusion medium, a modified Krebs–Henseleit solution (Bünger *et al.*, 1975), was maintained at 37 °C and gassed with O<sub>2</sub>/CO<sub>2</sub> (19:1). To ensure that the isolated heart was a non-working preparation, the mitral valve was cut to make it insufficient. Hearts were equilibrated by using constant-pressure perfusion for a minimum of 15 min; thereafter, perfusion was changed to constant flow (10 ml/min) and hearts were electrically paced at 290 beats/min. Coronary flow was measured with an electromagnetic flow meter (2434; Hellige, Freiburg, Germany), and perfusion pressure was monitored with a pressure transducer (P 23 ID; Statham, Oxnard, CA, U.S.A.). Radiolabelled compounds and the adenosine deaminase inhibitor EHNA were infused via the aortic cannula at a rate of either 50 or 100 μl/min. A second perfusion column, which contained Krebs–Henseleit solution equilibrated with O<sub>2</sub>/CO<sub>2</sub>/N<sub>2</sub> (6:1:3), was used to perfuse hearts with hypoxic medium.

At the end of all experiments hearts were freeze-clamped, freeze-dried and, after removal of connective and atrial tissue, extracted with 0.5 M-HClO<sub>4</sub> (5–10% wet wt./vol.). Acid extracts were neutralized with 2 M-KOH and the freeze-dried residue was redissolved in 2 ml of distilled water. A 150 μl sample was used for AdoHcy and adenosine determinations.

### Chromatography and radioactivity measurements

AdoHcy and adenosine were separated on a reversed-phase C<sub>18</sub> column with solutions (A) 0.25 mM-ammonium acetate buffer/10% (v/v) methanol, pH 5.0, and (B) methanol/water (2:1, v/v). Two pumps (M-45 and M 6000A; Waters Associates) were programmed (model 721 System Controller; Waters Associates) for gradient elution of samples injected (WISP 710B; Waters Associates), at a flow rate of 1.5 ml/min. The following elution conditions were used: 0–100% B, concave gradient, 8 min; 100% B, isocratic, 4 min; 100–0% B, linear, 1 min; total run length 15 min. Effluent was monitored by u.v. absorbance (model 441 absorbance detector; Waters Associates) at 254 nm. The column eluate was collected as appropriate, and the radioactivity measured by liquid-scintillation spectrometry (Philips PW 4700).

### Method I: measurement of transmethylation rate during normoxia

**Rationale.** Perfusion of isolated guinea-pig hearts with micromolar concentrations of [<sup>3</sup>H]adenosine and L-homocysteine reverses the AdoHcy hydrolase reaction towards net synthesis of AdoHcy (Schrader *et al.*, 1981), thereby labelling the intracellular pool of AdoHcy and increasing the AdoHcy tissue concentration. During perfusion with adenosine- and homocysteine-free medium (the post-labelling period), the AdoHcy hydrolyase reaction is again in the direction of net hydrolysis of AdoHcy, provided that adenosine and homocysteine are removed rapidly by further metabolism. This results in a decrease in the tissue content of AdoHcy and loss of radioactivity from this pool, although without necessarily any alteration in the specific radioactivity of AdoHcy.

Any decrease in the specific radioactivity of AdoHcy during this time reflects synthesis from a non-radioactive precursor source, this being AdoMet. Therefore the rate of decrease in the specific radioactivity of AdoHcy provides a measure of the rate of overall cellular transmethylation.

**Calculation.** After pre-labelling of the AdoHcy pool, there is a time-dependent decrease in tissue AdoHcy [AdoHcy(*t*)] and in the specific radioactivity of AdoHcy [*r*(*t*)]. Assuming there is no other source or drain of AdoHcy, the changes in AdoHcy pool size in the post-labelling period depend on both the rate (nmol/min per g) of AdoHcy formation from AdoMet (*V*<sub>1</sub>) and the rate of AdoHcy hydrolysis (*V*<sub>2</sub>):

$$\frac{d}{dt}\text{AdoHcy}(t) = V_1(t) - V_2(t) \quad (1)$$

Let *R*(*t*) be the amount of radioactive AdoHcy per g of heart (nmol/g), measured by its radioactivity (c.p.m./g). Let *r*(*t*) = *R*(*t*)/AdoHcy(*t*), measured in c.p.m./g ÷ nmol/g = c.p.m./nmol, denote the specific radioactivity of AdoHcy. Owing to continuous cellular transmethylation from unlabelled AdoMet, the specific radioactivity of AdoHcy decreases with time. From the definition of *r*(*t*):

$$R(t) = r(t) \times \text{AdoHcy}(t) \quad (2)$$

Assuming that every quantity *d*AdoHcy(*t*) = *V*<sub>2</sub>(*t*) × *dt* contains the same proportion of *r*(*t*) as is present in the AdoHcy pool, the rate of AdoHcy hydrolysis during any short interval *dt* from time *t* to time *t* + *dt* amounts to:

$$dR(t) = \frac{d}{dt}R(t)dt = -r(t)V_2(t)dt \quad (3)$$

From (2) and (3) follows:

$$\begin{aligned} -r(t)V_2(t) &= \frac{d}{dt}R(t) \\ &= \frac{d}{dt}r(t)\text{AdoHcy}(t) + r(t)\frac{d}{dt}\text{AdoHcy}(t) \\ V_2(t) &= -\frac{dr(t)/dt}{r(t)}\text{AdoHcy}(t) - \frac{d}{dt}\text{AdoHcy}(t) \end{aligned} \quad (4)$$

With (1) it follows that:

$$V_1(t) = V_2(t) + \frac{d}{dt}\text{AdoHcy}(t) \quad (5)$$

$$\begin{aligned} &= -\frac{dr(t)/dt}{r(t)}\text{AdoHcy}(t) \\ &= -\frac{d}{dt}\ln[r(t)]\text{AdoHcy}(t) \end{aligned} \quad (6)$$

**Method II: measurement of transmethylation rate during hypoxia**

**Rationale.** Hypoxic perfusion of isolated guinea-pig hearts increases the production rate of endogenous adenosine and, in the presence of micromolar concentrations of [<sup>35</sup>S]homocysteine, reverses the AdoHcy hydrolase reaction, resulting in the expansion and labelling of the AdoHcy pool with <sup>35</sup>S. Since homocysteine is the immediate precursor of AdoHcy, the

specific radioactivity of [<sup>35</sup>S]AdoHcy should be equivalent to that of [<sup>35</sup>S]homocysteine, provided that there is no other source of AdoHcy. AdoHcy, however, may also be synthesized from AdoMet, and therefore the specific radioactivity of [<sup>35</sup>S]AdoHcy will be less than that of [<sup>35</sup>S]homocysteine by an amount which reflects synthesis of AdoHcy from AdoMet. The dilution of the specific radioactivity of AdoHcy can then be used to determine the transmethylation rate.

**Calculation.** The following rate constants must be distinguished:



Under steady-state conditions, when the pool size of AdoHcy remains constant, the rate of hydrolysis of AdoHcy (*V*<sub>2</sub>) equals the rate of transmethylation (*V*<sub>1</sub>) plus the synthesis rate of AdoHcy from homocysteine and adenosine (*V*<sub>3</sub>):

$$V_2 = V_1 + V_3 \quad (7)$$

Under non-steady-state conditions, when the pool size of AdoHcy is increasing, owing to increased concentrations of the substrates adenosine and homocysteine, the rate of hydrolysis (*V*<sub>2</sub>) is less than the total synthesis rate of AdoHcy [*V*<sub>1</sub> + *V*<sub>3</sub>(1 + *X*)] and the rate of increase in the AdoHcy pool size is given by:

$$\frac{d}{dt}\text{AdoHcy} = V_1 + V_3(1 + X) - V_2 \quad (8)$$

where *X* designates the increase in *V*<sub>3</sub>.

Assuming that *V*<sub>1</sub> and *V*<sub>2</sub> remain unaltered from the steady state, and substituting *V*<sub>3</sub> = *V*<sub>2</sub> - *V*<sub>1</sub> [from (7)], then:

$$\frac{d}{dt}\text{AdoHcy} = XV_3 \quad (9)$$

If the AdoHcy pool is labelled with [<sup>35</sup>S]homocysteine, under near-steady-state conditions (no change in AdoHcy pool size), then the relative specific radioactivity of AdoHcy (*RK*) (expressed relative to the specific radioactivity of [<sup>35</sup>S]homocysteine) is determined by the relative rates of AdoHcy formation from AdoMet (*V*<sub>1</sub>) and from adenosine and [<sup>35</sup>S]homocysteine (*V*<sub>3</sub>):

$$\frac{V_1}{V_3} = \frac{1 - RK}{RK} \quad (10)$$

Similarly, if the AdoHcy pool is labelled under non-steady-state conditions (increasing AdoHcy pool size), the relative specific radioactivity of AdoHcy (*RK*<sub>1</sub>) is determined by the relative rates of AdoHcy formation from AdoMet (*V*<sub>1</sub>) and the increased rate from adenosine and [<sup>35</sup>S]homocysteine [*V*<sub>3</sub>(1 + *X*)]:

$$\frac{V_1}{V_3(1 + X)} = \frac{1 - RK_1}{RK_1} \quad (11)$$

From (10):

$$V_3 = \frac{V_1 \cdot RK}{1 - RK}$$

and substituting *V*<sub>3</sub> into (11) and solving for *V*<sub>1</sub>:

$$V_1 = \frac{XV_3(1 - RK_1 - RK + RK_1 \cdot RK)}{(RK_1 - RK)} \quad (12)$$

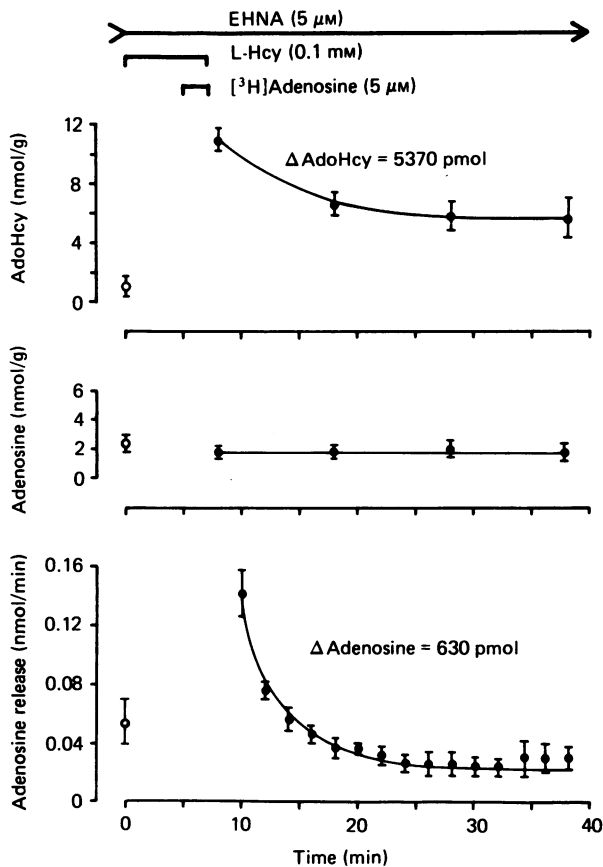


Fig. 2. Tissue AdoHcy and adenosine contents and coronary-venous adenosine release after pre-labelling of hearts with L-homocysteine and [ $^3\text{H}$ ]adenosine

Isolated guinea-pig hearts, electrically paced at 290 beats/min, were perfused at constant flow (10 ml/min) with a modified Krebs-Henseleit solution containing initially L-homocysteine thiolactone (0.1 mM) for 5 min, and then additionally [ $^3\text{H}$ ]adenosine for a further 2 min. Hearts were either freeze-clamped immediately or perfused for a further 10, 20 or 30 min. Results are mean values  $\pm$  s.e.m. ( $n = 4$  at each time point except 30 min, when  $n = 3$ ). Control values are represented by  $\circ$  ( $n = 6$  for control tissue contents of adenosine and AdoHcy;  $n = 4$  for control adenosine release).

## RESULTS

### Method I: transmethylation rate during normoxic perfusion

When isolated guinea-pig hearts were perfused with a normoxic medium (95%  $\text{O}_2$ ), tissue contents of adenosine, AdoHcy and AdoMet were  $2.1 \pm 0.1$ ,  $1.1 \pm 0.1$  and  $20.6 \pm 1.0$  nmol/g respectively ( $n = 6$ ). Normoxic hearts released adenosine at a rate of  $56 \pm 15$  pmol/min ( $n = 4$ ).

The intracellular AdoHcy pool was labelled by perfusing isolated hearts, at constant flow (10 ml/min), with medium containing  $5 \mu\text{M}$ -[ $^3\text{H}$ ]adenosine (final radioactivity  $5 \mu\text{Ci/nmol}$ ) and  $0.1 \text{ mM}$ -L-homocysteine thiolactone for a period of 2 min. EHNA ( $5 \mu\text{M}$ ), an adenosine deaminase inhibitor, was present throughout the experiment. Experiments were terminated by freeze-clamping either immediately after labelling (allowing for a 1 min wash-out) or after a further 10, 20 and 30 min perfusion period. Immediately after pre-labelling of

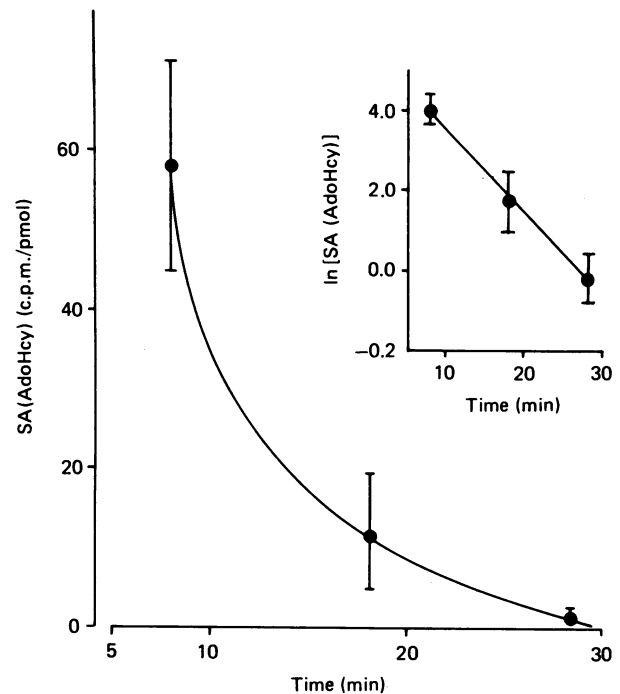


Fig. 3. Decrease in the specific radioactivity (SA) of AdoHcy after prelabelling of hearts with L-homocysteine and [ $^3\text{H}$ ]adenosine

Experimental conditions were as described in Fig. 2. Results are mean values  $\pm$  s.e.m. ( $n = 4$  for each time point except 28 min, when  $n = 3$ ). Inset displays the same data with logarithmic scale. [For further information see eqn. (6) under Method I.]

the tissue, the AdoHcy content was enhanced to  $10.9 \pm 0.7$  nmol/g. Global tissue adenosine contents remained unchanged, and cardiac adenosine release was increased to  $141 \pm 19$  pmol/min (Fig. 2). The specific radioactivity of AdoHcy was  $58 \pm 14$  c.p.m./pmol (Fig. 3). Tissue AdoMet contents were not significantly affected by the pre-labelling procedure, and AdoMet remained essentially unlabelled (results not shown). In the post-labelling period, both the AdoHcy tissue content and its specific radioactivity decreased: at 28 min the tissue content had decreased by 48% (Fig. 2) and the specific radioactivity of AdoHcy by 98% (Fig. 3). Tissue adenosine, however, remained constant (Fig. 2). Release of adenosine in the 30 min post-labelling period exceeded the control release by 630 pmol and was associated with a decrease in tissue AdoHcy equivalent to 5370 pmol of adenosine by the transmethylation pathway.

By using eqn. (5) above, the rate of transmethylation ( $V_1$ ) at 0, 10 and 20 min post-labelling was calculated to be 0.97, 1.35 and 0.62 nmol/min per g. Assuming an exponential decrease in the specific radioactivity of AdoHcy and by using values shown in the inset of Fig. 3,  $V_1$  was calculated to be 2.29, 1.38 and 1.19 nmol/min per g. However, since the pool size of AdoHcy also changed, the decrease in the specific radioactivity of AdoHcy may not have been truly exponential.

### Method II: transmethylation rate during hypoxic perfusion

The specific radioactivity of intracellular homocysteine was measured indirectly as follows: normoxic guinea-

**Table 1. Relative specific activity (RSA) values of AdoHcy and rate of AdoHcy accumulation during normoxic (95% O<sub>2</sub>) and hypoxic (30% O<sub>2</sub>) perfusion of isolated guinea-pig hearts with medium containing [<sup>35</sup>S]homocysteine thiolactone at either 7.5 μM (normoxia) or 50 μM (hypoxia)**

EHNA (5 μM) was present in all experiments.

	Normoxia	Hypoxia	
	RSA of AdoHcy (RK)	Rate of AdoHcy accumulation (XV <sub>3</sub> ) (nmol/min per g)	RSA of AdoHcy (RK <sub>1</sub> )
Median	0.26	1.38	0.65
Range	0.21–0.41	0.91–3.03	0.33–0.82
n	4	5	5

pig hearts were perfused with 100 μM-adenosine and 50 μM-L-[<sup>35</sup>S]homocysteine thiolactone (final radioactivity 1 mCi/mmol) for 10 min, which resulted in net synthesis of AdoHcy at a rate of 22.5 nmol/min per g (*n* = 4). This rate is 16 times the highest calculated rate of transmethylation (Method I: 1.35 nmol/min per g), which would result in a relative specific radioactivity of AdoHcy close to 1 (0.96). The measured specific radioactivity of [<sup>35</sup>S]AdoHcy in these experiments was 1.3 ± 0.1 c.p.m./nmol (*n* = 4), and all subsequent measurements of the specific radioactivity of AdoHcy were expressed relative to this value.

Measurement of the transmethylation rate during hypoxic perfusion (when endogenous adenosine formation is accelerated) was achieved by perfusing guinea-pig hearts for 10 min with medium equilibrated with 30% O<sub>2</sub> and containing 50 μM-L-[<sup>35</sup>S]homocysteine thiolactone (final sp. radioactivity 1 mCi/mmol). Experiments were terminated, after a 1 min wash-out period, by freeze-clamping. This procedure increased the tissue content of AdoHcy to 17.2 ± 3.9 nmol/g (*n* = 5), which is equivalent to a rate of AdoHcy formation (XV<sub>3</sub>; eqn. 9) of 1.7 ± 0.4 nmol/min per g (*n* = 5). The relative specific radioactivity of AdoHcy (RK<sub>1</sub>) in these experiments was 0.65 ± 0.10. The data are summarized in Table 1.

In a separate experimental series, guinea-pig hearts were perfused with normoxic medium containing a low concentration of [<sup>35</sup>S]homocysteine (7.5 μM). This procedure labelled the AdoHcy pool without altering the AdoHcy pool size. The lack of perturbation of the metabolic pool size indicates that these conditions were close to steady-state. The relative specific radioactivity of AdoHcy (RK) in these experiments was 0.26 (Table 1). By substituting the measured values of XV<sub>3</sub>, RK<sub>1</sub> and RK into eqn. (12), the rate of transmethylation (V<sub>1</sub>) during hypoxic perfusion was calculated to be 1.12 nmol/min per g.

## DISCUSSION

The present study provides the first quantitative data on the overall rate of transmethylation in the isolated guinea-pig heart. Previous studies have reported that the rate of protein methylation in the isolated rat heart is 0.7 nmol/min per g (Watkins & Morgan, 1979) and

that the rate of transmethylation in the liver *in situ* is 20 nmol/min per g (Hoffman, 1980). In the present study the transmethylation rate during normoxic perfusion of guinea-pig heart was estimated to be in the range of 0.62–1.35 nmol/min per g, mean value 0.98, which is equivalent to the rate of adenosine formation by this pathway. Since the rate of adenosine release by the isolated heart, measured in the venous effluent during normoxia, is only approx. 50 pmol/min per g (Fig. 2), the rate of adenosine production by the transmethylation pathway exceeds the basal release 15-fold, and strongly suggests that this pathway is an important intracellular source of adenosine. During hypoxic perfusion of the isolated heart, when release of adenosine into the venous effluent is over 1.5 nmol/min per g (Schradler *et al.*, 1977), transmethylation rate was determined to be 1.12 nmol/min per g. This suggests that the flux through the transmethylation pathway is not accelerated, and cannot explain the massive release of adenosine during hypoxic perfusion. Thus the fraction of adenosine derived from the transmethylation pathway during hypoxia is less important.

The measurement of transmethylation rate during normoxic perfusion (Method I) was based on the rate of dilution of a labelled AdoHcy pool. Ideally, studies on the turnover of a metabolite should be carried out under steady-state conditions, i.e. the incorporation of the label should not alter the pool size of the respective metabolite. In our experiments (Method I), this would have required perfusing hearts with low concentrations of adenosine of high specific radioactivity. However, adenosine at low concentrations (< 0.1 μM) is unlikely to reach the myocardial cells in the perfused heart, since this nucleoside is trapped by the high-affinity uptake system of the coronary endothelium (Nees *et al.*, 1985; Kroll *et al.*, 1987). In addition, adenosine kinase competes with AdoHcy hydrolase, thereby decreasing the labelling of the AdoHcy pool. In order to circumvent these problems, pre-labelling of the AdoHcy pool was carried out at relatively high [<sup>3</sup>H]adenosine and homocysteine concentrations (5 μM and 0.1 mM respectively), resulting in sufficient incorporation of label into the AdoHcy pool, but also expanding the AdoHcy pool size. In the post-labelling period, therefore, both the size of the AdoHcy pool and the specific radioactivity of AdoHcy decreased simultaneously. The mathematical analysis developed for this special case [see eqn. (5) in the Experimental section] takes into account the changing pool size and permits calculation of the rate of transmethylation for each data point in the post-labelling period.

Dilution of the labelled AdoHcy pool by using Method I has been assumed to be due solely to dilution from unlabelled AdoMet. The kinetic properties of AdoHcy hydrolase, however, make it possible that, despite continuous hydrolysis of AdoHcy, there is in addition synthesis of AdoHcy from adenosine and homocysteine. In this instance adenosine may be derived from the hydrolysis of 5'-AMP. This reaction would be equivalent to V<sub>3</sub> of eqn. (7) (see the Experimental section), and is referred to as the AdoHcy back-reaction. To our knowledge no estimate is available in the literature on the back-reaction when the net reaction is in the direction of hydrolysis of AdoHcy. Results obtained in the present study indicate that it could be as high as 35% of the transmethylation rate (see below). This value is likely to reflect an upper limit, as discussed below, but

the net effect would be to decrease the calculated transmethylation rate. Assuming the back-reaction to be 35% of the transmethylation rate, then the actual rate of transmethylation, measured by Method I, would be 0.64 instead of 0.98 nmol/min per g.

The measurement of transmethylation rate during hypoxia (Method II) was based on the specific radioactivity of [<sup>35</sup>S]AdoHcy relative to that of the intracellular precursor [<sup>35</sup>S]homocysteine, which, in turn, is determined by the relative rates of AdoHcy formation from AdoMet ( $V_1$ ) and from adenosine and homocysteine ( $V_3$ ). The increased rate of AdoHcy synthesis from adenosine and [<sup>35</sup>S]homocysteine was calculated by measuring the increase in AdoHcy which occurred with time. In order for this to reflect accurately the overall rate of synthesis, there should be no change in the hydrolysis rate of AdoHcy [this may occur because of an increase in precursor (AdoHcy) concentration]. An increased hydrolysis rate would cause an apparent decrease in transmethylation rate. Also, the calculation includes the measured back-reaction, which, if anything, is overestimated (see below). If  $V_3$  were 0 and not 35% of  $V_1$ , the transmethylation rate during hypoxia would be 0.90 instead of 1.12 nmol/min per g.

Perfusion of normoxic hearts with a low concentration of [<sup>35</sup>S]homocysteine (7.5 μM) permitted an estimation to be made of the AdoHcy hydrolase back-reaction. The estimation, which is expressed as a percentage of the transmethylation rate, was 35% and relied on measurements which indicated no change in the tissue concentration of AdoHcy. With the procedures used, control amounts of AdoHcy (1–2 nmol/g) were close to the limit of detection, and hence small increases in AdoHcy concentration could have occurred undetected. If there were a small increase in AdoHcy, this would decrease the calculated percentage of 35%, which should therefore be regarded as an upper limit.

Methyltransferases are inhibited by AdoHcy (Duerre, 1982). Measurements of transmethylation rate by both Methods I and II were undertaken when the tissue AdoHcy pool was expanded 10–15-fold. This increase is relatively moderate, since the AdoHcy pool size may increase by more than 100-fold (Schrader *et al.*, 1981; Deussen *et al.*, 1988). Also, no changes in AdoMet tissue contents were detected in these hearts. Nevertheless the possibility of partial inhibition cannot be entirely excluded.

Several important conclusions may be drawn from our data regarding the metabolism of adenosine in the normoxic and hypoxic heart. Firstly, the difference in transmethylation rate during normoxia (0.98 nmol/min per g) and venous release of adenosine (50 pmol/min per g) demonstrates that, quantitatively, the transmethylation pathway is an important intracellular source of adenosine. Secondly, since transmethylation is some 15-fold greater than the adenosine efflux rate, the adenosine formed by this pathway is largely salvaged or

metabolized intracellularly. Adenosine deaminase was inhibited by EHNA (5 μM) in all the experiments; therefore it is reasonable to assume that the greater proportion of AdoHcy-derived adenosine can be salvaged by adenosine kinase. Finally, hypoxic perfusion of the heart does not stimulate the transmethylation rate. This implies that the known hypoxia-induced increase in adenosine release is derived from 5'-AMP and that cellular transmethylation is a largely oxygen-independent process.

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