Compartmentalization of the Adenosine Pool of Dog and Rat Hearts

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SUMMARY. Studies in rat and dog hearts examined the hypothesis that the cardiac adenosine pool contains an intracellular compartment. Enzymatically dispersed rat cardiocytes contain 70 pmol adenosine/mg protein which is resistant to 10 U/ml adenosine deaminase (ADA). Incubating dog heart homogenates for 1 minute at 37°C with 10 U ADA/mL did not change adenosine levels perceptibly from the average control value of 1.28 nmol/g. Studies employing [3H]hypoxanthine arabinoside as an adenosine surrogate showed that this nucleoside penetrates into pericardial superfusates, attaining concentrations equal to those in blood plasma by 30 minutes. Since blood, cardiac interstitium, and pericardial superfusate are three compartments in series, this validates the use of pericardial superfusates equilibrated for ≥30 min as probes of cardiac interstitial composition. In eight dogs, pericardial superfusate adenosine concentration averaged 0.24 μM. Cardiac muscle adenosine content averaged 0.87 nmol/g, indicating that the interstitial compartment accounts for only 6% of the cardiac pool. Dog cardiac muscle contains a [3H]adenosine binding protein whose size, affinity for adenosine analogs, and ability to synthesize S-adenosylhomocysteine (AdoHcy) suggest it is S-adenosylhomocysteine hydrolase (SAH). Studies employing a dog erythrocyte model show that adenosine is bound to a protein in this cell; treatment with L-homocysteine greatly reduces the amount of adenosine recovered. The half-time for the dissociation of [3H]adenosine from SAH at 37°C is 2.5 hours, and in the presence of ADA is >6 hours. Thus, although adenosine bound to SAH can serve as a substrate for AdoHcy synthesis, this experiment does not support the idea that the dissociation of adenosine occurs to a physiologically significant extent. Thus, we are uncertain of the functional role of the intracellular adenosine compartment. (Circ Res 50: 617-626, 1982)

THE conventional view that the cardiac adenosine pool is exclusively extracellular is based on the supposition that the substantial adenosine deaminase and adenosine kinase activities in cardiac muscle would prevent intracellular accumulation of the nucleoside (Rubio and Berne, 1975; Olsson and Patterson, 1976). This one-compartment model was supported by the estimates of cardiac adenosine content then available, 0.2-0.3 nmol/g in the open-chest dog (Rubio and Berne, 1969; Olsson, 1970). The coronary vasoactivity of adenosine in such a preparation (Rubio et al., 1969; Olsson et al., 1979a) predicts that this amount of adenosine distributed in the cardiac interstitium could account for the coronary resistance typical of this preparation.

However, improved analytical techniques now yield much higher estimates of the size of the cardiac adenosine pool, as much as 1.5 nmol/g in the dog (Olsson et al., 1979b) and about 2 nmol/g in the guinea pig (Schrader et al., 1976). An adenosine pool this size would maximally dilate the coronary bed, even under basal conditions.

This crucial inconsistency in the evidence supporting a one-compartment model revived the hypothesis that the cardiac adenosine pool included an intracellular compartment (Kubler et al., 1970). Following the discovery that the enzyme S-adenosylhomocysteine hydrolase is an intracellular adenosine-binding protein (Hershfield and Kredich, 1978), this enzyme was shown to sequester adenosine in most tissues, including heart muscle (Ueland and Saebø, 1979). Recent studies by Schrader et al. (1981) and Schutz et al. (1981) have begun to define the physiological function of intracellular adenosine in guinea pig hearts, especially its role in controlling coronary vasomotion.

We report here experiments in dog and rat hearts and in dog red cells which further support a two-compartment model of the cardiac adenosine pool.

Methods

Adenosine deaminase (type 1), collagenase (type 1), catalase, hyaluronidase, aldolase, bovine serum albumin, l-homocysteine thiolactone and S-adenosylhomocysteine were from Sigma. Sephadex gels were from Pharmacia and Bio-Gels, Aminex resin and hydroxylapatite from Bio Rad. New England Nuclear supplied [8-14C]inosine, [8-14C]adenosine and [2,8,5'-3H]adenosine. [2-3H]Adenine arabinoside was from ICN, and was converted to the hypoxanthine analogue by incubation for 30 minutes at room temperature with 50 U adenosine deaminase in 1 ml 50 mM Tris-HCl, pH 7.5. Absorption of the nucleoside on a short column of octadecylsilyl silica gel (Sep-Pak, Waters Associates) sepa-
rated it from the enzyme and buffer salts, which were not retained. The product was eluted with 2 ml 20% ethanol and stored at -15°C. Immediately prior to use, aliquots of the stock solution were dried in vacuo to remove [3H]H2O resulting from exchange with solvent protons. An earlier report (Olsson et al., 1979a) lists the sources of adenosine analogues used to characterize the cardiac adenosine-binding protein.

Adenosine Assay

Samples for adenosine assay were extracted with 0.6 n HClO4 containing 5 x 10^4 dpm [3H]adenosine as a recovery standard. This extract was neutralized and adenosine separated by chromatography on 0.5 x 3 cm columns of Aminex A6 (Schrader et al., 1977). An aliquot of the eluate was counted to estimate recovery, and the remainder was dried in a stream of air and stored at -15°C until analysis. Adenosine was estimated by a competitive radioligand binding assay (Olsson et al., 1978b) which employed a nonlinear curve-fitting technique for standard curve refinement (Halch et al., 1976) and the system of Rodbard et al. (1968) for quality control. This technique detects ≥1 pmol of adenosine with a precision of 10-12%.

Adenosine in Enzymatically Dispersed Rat Cardiocytes

Male Sprague-Dawley rats weighing 150-200 g were anesthetized with pentobarbital, 50 mg/kg ip, and were given 1000 U heparin iv. The hearts were excised and immediately placed in ice-cold 0.14 M NaCl. Retrograde aortic perfusion was established with a 30°C solution containing (mg/ml): NaCl, 120; KCl, 5; KH2PO4, 1.2; MgSO4, 1.2; NaHCO3, 25; and glucose, 5.5. The perfusate was continuously gassed with 95% O2/5% CO2. Non-recirculating perfusion with this buffer for 5 minutes was followed by 1 hour recirculating perfusion with 70 ml of buffer containing 160 mg collagenase at a rate of 10 ml/min. The hearts were then minced and disrupted by aspirating tissue fragments into a pipet with a wide-bore tip. The fragments were filtered through a 350 μm nylon mesh and the cells in the filtrate were collected by centrifugation and washed twice in basic buffer containing 0.1 mM EDTA. Aliquots (3 ml) of the final suspension were incubated in buffer with or without adenosine deaminase, 10 U/ml, at 37°C in a rotating shaker gassed either with room air or 95% O2/5% CO2. After 1- or 5-minute incubations, the suspensions were treated with 3 ml ice-cold 0.6 n HClO4 and prepared for adenosine assay.

Cell counts, the ability to exclude 0.3% Trypan Blue, protein content (Lowry et al., 1951), and lactic dehydrogenase activity (Bergmeyer and Gawehn, 1975) in both the cells and medium characterized each suspension.

Adenosine in Pericardial Superfusates and Heart Muscle

Preliminary experiments assessed the rate at which nucleosides in the cardiac interstitium equilibrate with pericardial superfusates. The ideal tracer for this purpose would be a nucleoside which is not vasoactive, cannot be metabolized, does not penetrate cells, and is available in radiolabeled form. The biological properties of both the purine and sugar moieties of [3H]hypoxanthine arabinoside dictated its choice as the adenosine surrogate for these studies. Hypoxanthine nucleosides are vasoactive only at high concentrations, penetrate cells poorly, and are not substrates of either adenosine deaminase or adenosine kinase (Schnebli et al., 1967; Namn, 1973; Olsson et al., 1973, 1979a). Purine arabinosides are not vasoactive; neither do they readily penetrate cells (Olsson et al., 1973; Olsson et al., 1979a). Hypoxanthine arabinoside is an exceedingly poor substrate for purine nucleoside phosphorylase (Stoeckler et al., 1981). The radiolabeled nucleoside is readily prepared by the enzymatic deamination of commercially available [3H]adenine arabinoside.

Dogs premedicated with morphine (3 mg/kg sc) and anesthetized with 0.5-1.5% methoxyfluorane were prepared by insertion of femoral artery and vein catheters, bilateral nephrectomy and, through a right thoracotomy, insertion of a 6F catheter via the external jugular vein into the coronary sinus. The position of this catheter was verified by direct inspection and by sampling blood having a Po2 lower than that of right atrial blood. A soft polyvinyl catheter was inserted into the pericardial space and secured by a purse-string suture. A solution of 50 μCi [3H]hypoxanthine arabinoside was injected intravenously. After a 45-minute equilibration period, 0.75-1.0 ml/kg of 37°C 0.14 M NaCl was injected into the pericardial sac. Samples of arterial and venous blood and of superfusate were withdrawn 20, 40, 45, and 60 minutes later, centrifuged, and duplicate 0.5-ml samples of supernatant were mixed with 1.0 ml absolute ethanol. After separation of coagulated protein by centrifugation, duplicate 0.5-ml aliquots of supernatant were counted for 3H activity, employing the external standard ratio method of quench correction. Calculations of nucleoside concentration assumed a plasma-water content of 0.92 ml/ml and a superfusate water content of 0.99 ml/ml.

Comparisons of pericardial superfusate and myocardial adenosine levels used anesthetized, open-chest dogs fitted with a catheter in the pericardial space. Warmed (37°C) 0.14 M NaCl (0.5-1 ml/kg) was instilled into the pericardial space. Samples (1 ml) withdrawn 5, 10, 15, 20, and 30 minutes later were immediately mixed with 4 ml ice-cold 0.6 n HClO4. Immediately (<10 sec) after the last superfusate sample, the pericardium was opened widely and the left ventricle was sampled with metal tongs cooled in liquid N2. The muscle was pulverized in a percussion mortar cooled in liquid N2 and the purines extracted with 0.6 n HClO4 in preparation for adenosine assay. The product of superfusate adenosine concentration multiplied by interstitial space volume (0.21 ml/g; Frank and Langer, 1974) estimated the size of the interstitial adenosine compartment.

To assess the extent to which endogenous adenosine deaminase in the pericardial superfusate might destroy adenosine during the 30-minute period of equilibration, we collected superfusates equilibrated for 30 minutes from two dogs. To 1-ml aliquots, we added 0.1 nmol of [14C]adenosine and incubated the mixture for an additional 30 minutes. At the end of this interval, the reaction mixture was acidified with 50 μl 98% formic acid and percolated through a 0.7 x 4 cm column of Dowex 50-X8 (H+ form). After the column had been washed with 20 ml water, 10 ml of 2 n NH4OH-eluted adenosine, the ammonia eluate was dried in a stream of air and the residue was counted for 14C activity. This procedure separates inosine, which is not retained on DEAE cellulose, from adenosine, which binds under acid conditions and is eluted with ammonia. The recoveries of adenosine were 94-97%, indicating negligible adenosine deaminase activity in the pericardial superfusates.

Adenosine Deaminase-Resistant Adenosine in Heart Homogenates

This study employed the technique of Ueland and Saebø (1979). Samples of left ventricle from open-chest dogs ob-
tained by tongs cooled in liquid N₂ were pulverized in a percussion mortar cooled in liquid N₂. Microscopic examination showed the powder consisted almost entirely of amorphous debris. Few recognizable cells remained. In a 5°C room, part of the frozen muscle powder was weighed on a Cahn model 7500 balance and immediately mixed with 2 ml ice-cold 0.6 n HClO₄. The remaining 100-300 mg of muscle was weighed and placed in 5 ml of a solution of 50 U adenosine deaminase in 50 mM Tris·HCl, pH 7.5, which was kept in a waterbath at 37°C. A Polytron homogenizer run at half-speed for 15 seconds mixed the suspension of muscle powder. At 15 seconds and 1 minute later, 1 ml of the homogenate was mixed with 4 ml ice-cold 0.6 n HClO₄ and assayed for adenosine.

Preliminary experiments assessed the extent to which adenosine not bound to protein in vivo might become bound during incubation and thus escape deamination during incubation. A 10-μl aliquot of 0.1 mM [¹⁴C]adenosine was pipetted onto frozen muscle powder, after which the powder was homogenized in 5 ml of 37°C buffer containing ADA. Immediately after homogenization, 1 ml of ice-cold 1 n HClO₄ was mixed with the homogenate. Duplicate 1-ml aliquots of the supernatant separated by centrifugation were incubated at room temperature until chromatography of the second aliquot was complete (25-30 minutes). The fourth aliquot was mixed with 4 ml cold 0.6 n HClO₄ and the third aliquot was mixed with [¹⁴C]inosine and pumped over the Sephadex column. Protein (hemoglobin)-containing fractions of both column eluates were deproteinized with HClO₄, and aliquots of the subsequent fractions were counted for ¹⁴C activity. Each HClO₄ extract was prepared for adenosine assay.

Fresh dog ventricle was homogenized as described by Schrader et al. (1981) and centrifuged at 50,000 g for 30 minutes. The supernatant was concentrated approximately 10-fold by vacuum dialysis in a Schleicher and Schuell collodion thimble against 10 mM HEPES, pH 7.4, containing 1 mM 2-mercaptoethanol, 1 mM MgCl₂ and 0.01% activated charcoal. A 0.5-ml aliquot of dialyzed extract was filtered through a 0.9 X 50 cm column of Bio Gel A 1.5m. Aliquots of each fraction of eluate were assayed for 5'-adenosylhydroxycysteine synthetic activity (Schrader et al., 1981) and for the capacity to specifically bind [⁹⁹mCr]inosine.

Larger-scale preparations of adenosine-binding proteins in dog heart were carried out in a cold room and employed buffers supplemented with 2-mercaptoethanol and 0.01% NaN₃. Frozen heart muscle (up to 1 kg) was homogenized in 5 ml/g 10 mM NaHPO₄, pH 7.5. The supernatant separated by centrifugation for 30 minutes at 16,500 g was filtered through eight layers of cheese-cloth and stirred during the portionwise addition of 342 g/liter (NH₄)₂SO₄. Protein precipitating over the next 4 hours was dissolved in and dialyzed against 5 mM Tris·HCl, pH 7.4. This solution was pumped onto a 5 X 50 cm column of DEAE-cellulose equilibrated with dialysis buffer. The column was washed with this buffer until the absorbance of the effluent was <0.01 and then was developed with a linear NaCl gradient formed from 2 liters each of starting buffer and 0.5 M NaCl. Fractions containing [⁹⁹mCr]inosine binding activity were pooled, concentrated by precipitation with 342 g/liter (NH₄)₂SO₄. The protein collected by centrifugation was dissolved in and dialyzed against 10 mM KHPO₄, pH 7.0, and applied to a 2.5 X 20 cm column of hydroxyapatite equilibrated with dialysis buffer. The column was washed with this buffer and then developed with a linear gradient formed from 0.5 liter each of starting buffer and 0.5 M KHPO₄, pH 7.0. Fractions containing [⁹⁹mCr]inosine-binding activity were pooled and concentrated by (NH₄)₂SO₄ precipitation. The protein was dissolved in a minimum of 0.1 M NaHPO₄, pH 7.0, in 0.2 M NaCl and 10 ml aliquots were filtered through a 2.6 X 100 cm column of Bio Gel A 1.5 m which had been equilibrated with the NaHPO₄/NaCl buffer. Fractions containing [⁹⁹mCr]inosine-binding activity were pooled and frozen.

Estimates of molecular weight by the method of Siegel and Monty (1966) employed gel filtration through a 0.9 X 50 cm column of Bio Gel A 1.5 m calibrated with proteins of known Stokes radius and sedimentation through isokinetic sucrose gradients (McCart, 1968).

Adenosine-Binding Proteins in Red Cells and Heart Muscle

Dog blood was centrifuged, the buffy coat and supernatant plasma aspirated, and the cells suspended in 5 volumes of cold 0.14 M NaCl. After five washes, the addition of 4 volumes of 2 M MgCl₂ lysed the cells. The hemolysate was centrifuged to separate stroma, and four 2-ml aliquots of the supernatant were removed. The first was placed directly in 4 ml ice-cold 0.6 n HClO₄. The second was mixed with 2 X 10⁶ dpm [¹⁴C]inosine, which served as a total volume marker, and was pumped through a 1.6 X 20 cm column of Sephadex G25 equilibrated and eluted at 4°C with 2 M MgCl₂ in 50 mM Tris·HCl, pH 7.5. The third aliquot was mixed with 20 μl 0.1 M l-homocystein in 1 M NaHPO₄, pH 7, freshly prepared by hydrolysis of the thiolactone in 1 n NaOH for 10 minutes, followed by neutralization with an equal volume of 1 M NaHPO₄. The fourth aliquot, which served as a control for the third, was incubated at 37°C and 500 μl was filtered through eight layers of cheese-cloth and stirred during the portionwise addition of 342 g/liter (NH₄)₂SO₄. Protein precipitating over the next 4 hours was dissolved in and dialyzed against 5 mM Tris·HCl, pH 7.4. This solution was pumped onto a 5 X 50 cm column of DEAE-cellulose equilibrated with dialysis buffer. The column was washed with this buffer until the absorbance of the effluent was <0.01 and then was developed with a linear NaCl gradient formed from 2 liters each of starting buffer and 0.5 M NaCl. Fractions containing [⁹⁹mCr]inosine binding activity were pooled, concentrated by precipitation with 342 g/liter (NH₄)₂SO₄. The protein collected by centrifugation was dissolved in and dialyzed against 10 mM KHPO₄, pH 7.0, and applied to a 2.5 X 20 cm column of hydroxyapatite equilibrated with dialysis buffer. The column was washed with this buffer and then developed with a linear gradient formed from 0.5 liter each of starting buffer and 0.5 M KHPO₄, pH 7.0. Fractions containing [⁹⁹mCr]inosine-binding activity were pooled and concentrated by (NH₄)₂SO₄ precipitation. The protein was dissolved in a minimum of 0.1 M NaHPO₄, pH 7.0, in 0.2 M NaCl and 10 ml aliquots were filtered through a 2.6 X 100 cm column of Bio Gel A 1.5 m which had been equilibrated with the NaHPO₄/NaCl buffer. Fractions containing [⁹⁹mCr]inosine-binding activity were pooled and frozen.

Adenosine-Binding Activity

This method has been described previously (Olsson, 1978). Briefly, mixtures (0.1 ml) containing 50 mM tris·HCl, pH 7.4, 40 μM EHNA, 5 X 10⁶ dpm [²,⁸,⁵⁻³H]adenosine, and protein diluted so that adenosine binding was proportional to protein concentration, were incubated at 0°C for 4-6 hours, or, to assure binding equilibrium, occasionally as long as 24 hours. The samples were treated with 25 μl charcoal-dextran-albumin to absorb unbound adenosine, diluted with 1 ml 50 mM Tris·HCl, pH 7.4, and centrifuged.
for 15 minutes at 700 g to separate the charcoal. An aliquot of the supernatant was counted for \[^{3}H\] activity.

**Adenosine-Binding Capacity of Cardiac Muscle**

Samples of left ventricle weighing 10-15 g were homogenized with a Polytron in 9.2 ml/g 10 mM KHPO\(_{4}\), pH 7.0, for 15 seconds at half-maximum speed. Aliquots were further homogenized by hand with a Dounce homogenizer which had a tightly fitting Teflon pestle. The supernatant obtained by centrifugation for 1 hour at 96,000 g av, which represented a 1:10 dilution of the "soluble" heart proteins, was diluted serially to a final concentration of 1:640. Aliquots of each dilution were then assayed for \[^{3}H\]adenosine-binding capacity. In this instance, each reaction mixture contained 18.9 pmol \[^{3}H\]adenosine.

**Data Analysis**

Two-way analysis of variance for repeated measures evaluated the results of the pericardial superfusion studies and of the effect of adenosine deaminase on the adenosine content of heart homogenates. A Student's t-test for paired samples was used for other evaluations. Group data are reported as mean ± SEM.

**Results**

**Adenosine in Enzymatically Dispersed Rat Cardiocytes**

The rats cardiocyte suspensions contained an average of 5.4 \(\times\) 10\(^4\) cells/ml and 1.3 mg protein/ml. Although 89% of these cells excluded trypan blue, >50% contracted on exposure to 1 mM Ca\(^{++}\), indicative of sarcolemmal damage during isolation. Preliminary experiments employing four suspensions of cells in buffer containing 10 U/ml adenosine deaminase showed that 99.7% of exogenous \[^{14}C\]adenosine added to a final concentration of 10 mM was degraded to \[^{14}C\]inosine in <5 seconds.

The total adenosine content of the cell suspension from an individual animal varied between 700 and 500 pmol/mg protein after 1 minute of incubation and rose approximately 2-fold by 5 minutes of incubation, \(P < 0.01\). The initial adenosine concentration was unrelated to whether the cells were incubated in air or in 95% O\(_2\)/5% CO\(_2\). The adenosine concentrations in six cardiocyte suspensions in buffer containing adenosine deaminase gassed with 95% O\(_2\)/5% CO\(_2\) averaged 72 ± 9.5 pmol/mg protein after 1 minute and 63 ± 10 pmol/mg protein after 5 minutes. In contrast, cells from seven different animals, suspended in buffers containing deaminase but incubated in room air, exhibited a significant time-dependent increase in adenosine from a 1-minute value of 70 ± 23 pmol/mg protein to a 5-minute value of 202 ± 69 pmol/mg protein, \(P < 0.02\). In neither of these experiments, nor in any of over 50 preliminary experiments, did adenosine deaminase ever completely destroy the adenosine in a cell suspension.

**Adenosine in Dog Myocardium and Pericardial Superfusates**

Figure 1 summarizes experiments in six dogs assessing the penetration of \[^{3}H\]hypoxanthine arabinoside into the pericardial space. During the 40 minutes of observation, arterial and venous plasma \[^{3}H\] activity differed by <2%, and neither declined significantly during this interval. After 20 minutes of equilibration, the tracer concentration in the pericardial superfusate was significantly lower than that in coronary plasma, \(P < 0.01\), but differed insignificantly after that time.

Figure 2 and Table 1 summarize the comparisons, in eight dogs, of adenosine concentration in pericardial superfusates and in heart muscle. Pericardial fluid adenosine concentration rose steadily during equilibration to reach values which were essentially constant after 20 minutes. Although the values for individual dogs ranged between 0.11 and 0.36 \(\mu\)M, the difference between the 20- and 30-minute estimates was not significant. The adenosine concentration in cardiac muscle samples ranged between 0.71 and 1.27 pmol/mg protein.
nmol/g, averaging nearly 4 times that in superfusate. In these experiments, interstitial adenosine averaged 6 ± 2% of the cardiac pool.

**Adenosine Deaminase-Resistant Adenosine in Dog Heart Homogenates**

This experiment employed samples of left ventricle from five open-chest dogs. The adenosine content of heart muscle powder placed directly into HClO₄, averaged 1.28 ± 0.17 nmol/g. The adenosine content of muscle powder incubated with adenosine deaminase for 15 seconds averaged 1.37 ± 0.23 nmol/g and for 60 seconds averaged 1.28 ± 0.17 nmol/g.

**Adenosine Binding Protein in Dog Red Cells and Heart Muscle**

The adenosine content of freshly hemolyzed erythrocytes from six dogs averaged 187 ± 14 pmol/ml packed cells. Chromatography of the hemolysate clearly separated adenosine, which emerged in protein-containing fractions, from the [14C]inosine used as a total volume marker. The 162 ± 18 pmol adenosine/ml-packed cells found in these fractions represents an 87% recovery. Incubating the hemolysate for up to 30 minutes at room temperature reduced adenosine content insignificantly to 178 ± 17 pmol/ml packed cells. However, treating the hemolysate for a comparable period with t-homocysteine reduced the recovery of adenosine to 42 ± 6 pmol/ml packed cells.

Based on this evidence that adenosine is bound to S-adenosylhomocysteine hydrolase, we next examined the susceptibility of this adenosine to deamination by adenosine deaminase. This experiment employed erythrocyte "adenosine analog-binding protein" (Olsson, 1978) which we subsequently identified as S-adenosylhomocysteine hydrolase (Olsson, unpublished data). The dissociation of the complex of this enzyme with [3H]adenosine is actually slower in the presence of adenosine deaminase than when dissociation of the complex is promoted by incubation with charcoal (Fig. 3). This experiment indicates that, once bound to this enzyme, adenosine is resistant to deamination.

**FIGURE 3.** Dissociation of [3H]adenosine from S-adenosylhomocysteine hydrolase. A mixture of 0.4 mg rabbit erythrocyte cytosol and 8 × 10⁵ dpm [3H]adenosine in 4 ml 50 mM Tris-HCl was incubated 4 hours at 0°C. Unbound ligand was removed with 0.1 ml charcoal-dextran. The concentration of bound ligand was now 16, 370 dpm/50 µl equivalent to 3.7 nM. Aliquots (2 ml) of the supernatant were treated with 25 µl of charcoal-dextran or with 0.2 ml Tris buffer containing 50 mM adenosine deaminase. At hourly intervals, duplicate 50-µl aliquots of each mixture were treated with 25 µl of charcoal-dextran, diluted with 1.0 ml cold Tris and, after centrifugation, 0.7-ml aliquots of the supernatant were counted. The amount of bound ligand remaining is expressed as a percentage of the activity at time = 0. Note that dissociation in the presence of charcoal (•) has a half-time slightly over 2.5 hour, but in the presence of adenosine deaminase (○) the half-time is > 6 hours.
The specific binding of $[^{14}C]$adenosine in serially diluted heart muscle extracts estimated the adenosine-binding capacity of intact heart. Binding was proportional to protein concentration only in relatively dilute extracts (Fig. 5). The reason for inhibition of binding in more concentrated extracts is uncertain, but could be due to, for example, incomplete inhibition of adenosine deaminase or dissociation of the enzyme-ligand complex in accordance with the law of mass action. Estimates of adenosine-binding capacity of four hearts averaged $2.9 \pm 0.7 \text{ nmol/g}$.

**Discussion**

These experiments independently confirm observations by others which establish the existence of an intracellular adenosine compartment in cardiac muscle. Ueland and Saebø (1979) demonstrated adenosine deaminase-resistant adenosine in many organs of the rat, including heart muscle. Resistance to deamination was due to the binding of adenosine to a protein (Ueland and Døskeland, 1977) subsequently identified as the enzyme S-adenosylhomocysteine hydrolase (Saebø and Ueland, 1978). The adenosine concentration of hepatocytes suspended in adenosine deaminase has served as a means for estimating the size of the intracellular adenosine compartment in this tissue (Belloni et al., 1980). Our experiments with enzymatically dispersed rat cardiocytes treated with adenosine deaminase show that adenosine does exist inside heart cells. The size of this compartment does not change over 5 minutes in cultures equilibrated with 95% O$_2$, but it increases nearly 3-fold in less well-oxygenated cultures equilibrated with air. We recognize that the sarcolemma of these cells was damaged during exposure to collagenase/hyaluronidase, as evidenced by abnormal sensitivity to Ca$^{++}$. However, we believe that—although these cells are probably too damaged for detailed studies of purine metabolism—they are still suitable for the limited observations made in this particular experiment. “Leaky” cells should be more permeable to adenosine deaminase, so that it is significant to find adenosine

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$^*$ Reaction mixtures consisted of $8.5 \mu$g protein, $72,500 \text{ dpm} [^{3}H]\text{adenosine (1 pmol)},$ and $10^{-5}$M analog in $100 \mu$l $50 \text{ mm Tris-HCl, pH 7.5.}$ After 4 hours at 0°C, the mixtures were processed as described in the text. In the absence of analog the protein bound 24,900 dpm. Apparent binding was corrected for nonspecific binding of 1,490 dpm.
under conditions in which the deaminase might have enhanced access to the cell interior. Our observation that adenosine deaminase does not change the amount of adenosine recovered from heart homogenates also confirms the experiments of Ueland and Saebo. More importantly, it validates our experiments with dispersed cardiocytes by showing that, irrespective of the state of the sarcolemma, adenosine inside cells is resistant to deamination. The fact that deaminase-resistant adenosine accounted for all the adenosine present in the cardiac homogenates independently confirms the estimates of compartment sizes made by comparing adenosine levels in heart muscle and pericardial superfusates. The comparison of the adenosine levels in heart muscle incubated with adenosine deaminase for 60 seconds with the levels in frozen muscle powder placed directly in cold HClO4 entails an analytical error of ~12%. The pericardial superfusate experiments estimate that extracellular adenosine, which should be susceptible to deamination, accounts for only 6% of the cardiac pool, a difference too small to be detected in the heart homogenate experiment by our adenosine assay method.

The use of pericardial superfusates to probe the composition of the cardiac interstitium is valid only if the two compartments are in equilibrium. Because available evidence (Miller et al., 1980) does not establish that such an equilibration occurs, it was necessary to define the conditions under which this experimental technique might be applicable. The experiments employing [3H]hypoxanthine arabinoside as an adenosine surrogate show that, by the time superfusate has been in contact with the heart for 30 minutes, superfusate and plasma tracer concentrations are equal. The blood plasma, cardiac interstitium, and pericardial superfusate constitute three compartments in series. Since the tracer concentrations in plasma and superfusate were equal by 30 minutes of equilibration, the concentration in the interstitial compartment was, of necessity, equal to that in the superfusate. Thus, comparisons of adenosine in superfusates and heart muscle after this length of equilibration are appropriate. Interestingly, pericardial superfusate adenosine concentrations appear to reach stable values even faster than [3H]hypoxanthine arabinoside. The accuracy of the adenosine assay undoubtedly contributes to this result by obscuring any real differences which might exist between the 20th and 30th minute of equilibration. On the other hand, it is possible that the continuous production of adenosine by subepicardial cardiocytes could actually accelerate equilibration. Although adenosine in the superfusate probably originates in only the most superficial layers of the heart, normally oxygenated heart muscle does not exhibit a transmural gradient of adenosine concentration (Foley et al., 1979). Consequently, the adenosine concentration of the subepicardial interstitium probably represents that of the entire interstitium fairly well. Of far greater concern is that the pericardial superfusate is an open compartment, i.e., adenosine can leave this space by routes other than diffusion back into the left ventricular interstitium. Losses through these alternate routes will prevent the adenosine concentration in the superfusate from reaching the same level as that in the interstitial space. One route of departure could be via deamination, but our assays of superfusate adenosine deaminase activity show that this is negligible. This observation is similar to that of Miller et al. (1980). A second route of loss could be via the atria and right ventricle, whose interstitial adenosine concentrations are completely unknown but whose surfaces constitute an important fraction of the total area available for adenosine exchange. If the interstitial adenosine concentrations in these chambers is lower than that of the left ventricle, this would tend to remove adenosine from the superfusate and lead to an underestimation of its concentration in the left ventricular interstitium. We know of no practical way to assess this probable error. Finally, adenosine might be lost across the pericardial membrane, another possibility which we do not know how to test. We believe, however, that neither incomplete equilibration, the possibility of a transmural adenosine gradient, nor the likelihood that the adenosine concentration in superfusates may not represent that of left ventricular interstitium are collectively important enough to challenge the conclusion that the interstitial compartment constitutes only a small fraction of the left ventricular adenosine pool. This conclusion is independently supported by the failure of adenosine deaminase to materially alter the adenosine concentration in heart muscle homogenates.

A model of cardiac adenosine metabolism developed by Manfredi and Sparks (1981) additionally supports our estimate of the size of the interstitial adenosine compartment. This model is based on these authors' measurements of the rate of adenosine release into coronary venous blood and literature data on rates of cellular uptake, capillary pore size, and diffusion coefficient of adenosine. It predicts an interstitial adenosine concentration of about 0.15 μM.

Gel filtration of cardiac muscle extracts shows that [3H]adenosine binding and S-adenosylhomocysteine hydrolase catalytic activities have the same inclusion volume, suggesting that both may be properties of the same protein. Several laboratories have demonstrated the high-affinity binding of adenosine to the S-adenosylhomocysteine hydrolase of rat liver and human placenta (Hershfield and Kredich, 1978), mouse liver (Ueland and Deskeland, 1977), and rabbit erythrocyte (Olsson, 1978). We found that the adenosine-binding protein had a molecular weight of about 180,000, similar to that of the "adenine analog-binding protein" of rat heart muscle (Sugden and Corbin, 1976) and the S-adenosylhomocysteine hydrolases of mouse liver [180,000 (Ueland and Deskeland, 1977)] and beef liver [192,000 (Palmer and Abeles, 1979)]. As shown here, the binding protein of dog heart exhibits a high affinity for adenosine. The extent to which adenosine analogs inhibit the binding of [3H]adenosine is quite similar to that of the rabbit eryth-
rocyte "adenine analog-binding protein" (Olsson, 1978), later shown to be $S$-adenosylhomocysteine hydrolase. We earlier reported the existence of two additional adenosine-binding proteins in heart muscle, designated L and S (Olsson et al., 1979b). We now believe that these proteins are artefacts, probably the result of dissociation and recombination of sub-units of the major protein during purification.

Schrader et al. (1981) perfused isolated guinea pig hearts with buffers containing L-homocysteine thiolactone and found that this treatment promoted the accumulation of $S$-adenosylhomocysteine. Under hypoxic conditions, this treatment inhibited the expected rise in adenosine concentration, probably through conversion of adenosine to $S$-adenosylhomocysteine as fast as the adenosine was formed. Curiously, perfusion with this amino acid did not lower the adenosine content of normally oxygenated heart, even though $S$-adenosylhomocysteine levels rose. There are three possible explanations for this result, the first being that adenosine was not bound to this enzyme. The second is that the rates of penetration and hydrolysis of L-homocysteine thiolactone were too slow, relative to the rates of adenosine production, to effect a net decrease in the amount of adenosine bound to the enzyme. The third possibility is that the intracellular compartment consists of metabolically inert adenosine bound to the enzyme through "suicide inactivation" (Hershfield et al., 1979). Since this adenosine would be bound to an irreversibly inactivated enzyme, it could not serve as a substrate for $S$-adenosylhomocysteine synthesis, but the fraction of enzyme still in the native state would be able to catalyze the conversion of newly formed adenosine to $S$-adenosylhomocysteine.

In our experiments testing the hypothesis that adenosine bound to $S$-adenosylhomocysteine hydrolase can serve as a substrate for the synthesis of $S$-adenosylhomocysteine, we employed red cell homolysates as a simple model of the cardiac cytosol. Gel filtration showed that all the adenosine in these hemolysates was associated with proteins. Treatment of these hemolysates with L-homocysteine greatly reduced adenosine recovery, indicating that adenosine is bound to $S$-adenosylhomocysteine hydrolase which retains catalytic activity. This excludes the possibility that suicide inactivation is an important consequence of adenosine binding in vivo. Thus, the simplest explanation for the failure of homocysteine thiolactone to lower adenosine levels in the oxygenated hearts is that the rates of penetration and hydrolysis of the thiolactone limit the production of $S$-adenosylhomocysteine.

We estimate that the adenosine-binding capacity of dog heart is only slightly lower than that calculated for guinea pig heart by Schrader et al. (1981). This is somewhat surprising, since these authors found that $S$-adenosylhomocysteines hydrolase activity of guinea pig heart is about 16 times higher than that of dog heart. Some of this discrepancy may be due to their use of an estimate of molecular weight, 235,000 (Richards et al., 1978), higher than that found by others and also to the assumption that $S$-adenosylhomocysteine hydrolase, which contains four apparently identical subunits (Palmer and Abeles, 1979), binds only 2 mol adenosine/mol enzyme.

The compartmentalization of the cardiac adenosine pool has several important consequences. Foremost of these is that, since only a small fraction of the cardiac pool participates directly in coronary vaso-motion, tests of the adenosine hypothesis which rely on comparisons of coronary resistance with total adenosine content may be invalid. The reliability of such evidence reported from this and other laboratories depends on whether the sizes of the two adenosine compartments change concordantly. This crucial information is not yet available.

The interstitial adenosine concentration estimated here, 0.24 $\mu$M, is at or below the threshold concentration of the coronary vasoactivity of exogenous adenosine in similar open-chest dog preparations (Olsson et al., 1978a, 1979a). This is consistent with the observation that intravenous infusion of adenosine deaminase and/or theophylline do not alter coronary resistance (Saito et al., 1981), and suggests that adenosine contributes little to "basal" coronary resistance.

The physiological function of adenosine in the intracellular compartment is unclear. Schutz et al. (1981) found that treating hypoxic hearts with L-homocysteine curtails both the accumulation within and release of adenosine from these hearts. They proposed that adenosine bound intracellularly to $S$-adenosylhomocysteine hydrolase contributes at least part of the adenosine released from hypoxic hearts. This interpretation, while plausible, depends on the extent to which the dissociation of the $[S$-adenosylhomocysteine hydrolase-adenosine] complex is able to generate free adenosine. Available evidence, which is limited, does not support this hypothesis. Essentially, all the adenosine in heart muscle homogenates and a substantial fraction of the adenosine in other organs (Ueland and Sæbø, 1979) is unaffected by one to several minutes' exposure to adenosine deaminase. This suggests that this adenosine dissociates only slowly from $S$-adenosylhomocysteine hydrolase. The present experiments show this directly. Dissociation of the adenosine-S-adenosylhomocysteine hydrolase complex is an unimolecular reaction, i.e., the rate of adenosine release is proportional to the concentration of the enzyme-ligand complex. Our in vitro studies of the dissociation in the presence of charcoal, an efficient adenosine trap, define the maximum rate for this process. The $t_{1/2}$ of dissociation is about 2.5 hours, equivalent to a rate constant of ln 0.5/150 min, or 0.00466/min. Thus, the rate at which adenosine will be released from the $\sim 1 \text{ nmol adenosine/g heart muscle will be only about } 5 \text{ pmol/min, which is far below the rates of adenosine release, for example, during cardiac hypoxia. Adenosine binding is characterized by a high } K_D, \text{ which means that in the presence of a less efficient adenosine trap, any adenosine released is likely to be bound again to the enzyme. This probably explains why adenosine deaminase, which has a relatively low affinity for aden-}
protein (K_m = 40 μM) was less effective than charcoal in promoting dissociation of adenosine from its complex with S-adenosylhomocysteine hydrolase. Other enzymes with a higher affinity for adenosine, e.g., adenosine kinase, whose K_m is <1 μM, or the adenosine transport carrier, whose K_m is about 10 μM (Olsson et al., 1972), would undoubtedly compete more effectively than adenosine deaminase. However, since charcoal promotes the dissociation of the [S-adenosylhomocysteine hydrolase-adenosine] complex as effectively as adenosine itself (Olsson, 1978), the rate of dissociation in the presence of charcoal is indeed a maximum rate. Thus, although our red cell studies confirm that adenosine bound to this enzyme can readily be converted to S-adenosylhomocysteine, we know of no evidence that the dissociation of adenosine is quantitatively significant. Finally, although S-adenosylhomocysteine hydrolase certainly can catalyze the hydrolysis of S-adenosylhomocysteine, which Schrader et al. (1981) have identified in heart muscle, it does so at appreciable rates only when the reaction is forced by high concentrations of substrate and when the adenosine released is trapped by being converted to something else, e.g., to inosine by adenosine deaminase. In this case, of course, the ultimate product of dissociation is not adenosine but, rather, one of its metabolites. The possibility that the binding of other cell metabolites, metal ions, etc., might allosterically promote the dissociation of adenosine from this enzyme under physiological conditions is unexplored.

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