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Measurement of Plasma Adenosine Concentration: Methodological and Physiological Considerations[1] (42523)

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Abstract. This study tested the hypothesis that measurements of plasma adenosine concentration made on samples of blood obtained in dipyridamole and EHNA (i.e., "stopping solution") may be falsely elevated as a result of ongoing *in vitro* production and accumulation of adenosine during sample processing. Studies were performed with samples of anticoagulated blood obtained from anesthesized domestic swine. Adenosine concentration of ultra filtrated plasma was determined by high-pressure liquid chromatography (HPLC). The following parameters were evaluated: (i) rate of clearance of [^{3}H]adenosine added to plasma, (ii) endogenous adenosine concentration of matched blood samples obtained in "stopping solution" alone, "stopping solution" plus EDTA, and perchloric acid (PCA), (iii) plasma and erythrocyte endogenous adenosine concentration in nonhemolyzed samples, and (iv) plasma adenosine concentration of samples hemolyzed in the presence of "stopping solution" alone or "stopping solution" plus EDTA. We observed that (i) $\geq$95% of [^{3}H]adenosine added to plasma is removed from it by formed elements of the blood in <20 s, (ii) plasma adenosine concentration of samples obtained in "stopping solution" alone is generally 10-fold greater than that of matched samples obtained in "stopping solution" plus EDTA, (iii) deliberate mechanical hemolysis of blood samples obtained in "stopping solution" alone resulted in substantial augmentation of plasma adenosine levels in comparison with matched nonhemolyzed specimens—addition of EDTA to "stopping solution" prevented this, and (iv) adenosine content of blood samples obtained in PCA agreed closely with the sum of plasma and erythrocyte adenosine content of samples obtained in "stopping solution" plus EDTA. The data obtained demonstrate that (i) plasma adenosine concentrations are falsely elevated in samples of blood obtained in "stopping solution" alone, and (ii) addition of EDTA to "stopping solution" blocks *in vitro* production and accumulation of adenosine. Finally, rapid removal of adenosine from plasma by formed elements of blood may make it difficult to employ measurements of plasma adenosine concentration to assess physiological processes even in the absence of *in vitro* production of the nucleoside.

Measurements of plasma endogenous adenosine concentration are complicated by the fact that the nucleoside may be removed from plasma by formed elements of the blood and subsequently either deaminated or rephosphorylated (1, 2). In order to prevent loss of adenosine from plasma in blood samples obtained in the course of physiological studies, previous investigators have adopted the practice of withdrawing blood directly into syringes containing a small volume of dipyridamole alone (3, 4) or dipyridamole and erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) (5, 6). Dipyridamole in the "stopping solution" is employed to inhibit cellular nucleoside transport (7, 8), and EHNA is used to inhibit adenosine deaminase activity (9). The "stopping solution" method for preserving adenosine in plasma assumes (i) that removal of adenosine added to the plasma is relatively slow compared with the time required to withdraw blood from its collection site and place it in the "stopping solution," and (ii) that once in contact with the "stopping solution" *in vitro* loss or production of adenosine does not occur. However, metabolic activity of formed elements of the blood is not necessarily abolished during handling and processing of the sample. Thus, ongoing consumption of ATP and pro-

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duction of adenosine in the presence of dipyridamole and EHNA could lead to substantial accumulation of adenosine in the sample. Accordingly, the present investigation was performed to test the hypothesis that plasma adenosine levels may be falsely elevated in samples of blood obtained in "stopping solution" alone.

Materials and Methods. *1. Blood sampling procedure.* Venous blood was obtained from anesthetized, closed chest, domestic swine through a 7 French angiographic catheter (length 130 cm) which was positioned in the inferior vena cava. The catheter was cleared of residual, stagnant blood prior to obtaining samples for analysis. Once cleared, 3 ml of blood was aspirated into a chilled 5-ml syringe containing heparin (50 IU in 0.05 ml) plus 0.55 ml of either "stopping solution" (26 μM EHNA, 100 μM dipyridamole in normal saline) or "stopping solution" plus Na-ethylenediaminetetraacetic acid (EDTA, 4 mM in normal saline) or 8% perchloric acid (PCA). Concentrations are for the solutions themselves and not the final reaction mixture. Approximately 10–15 sec were required to draw the blood through the catheter into the syringe. The syringe was then capped and inverted back and forth gently several times to help ensure complete mixing of the "stopping solution" or PCA with the whole blood. Immediately thereafter, the syringe was placed on ice pending further processing. Preweighed syringes were employed and the hematocrit of each sample was determined in order to accurately measure plasma volume of each sample. The syringe containing the whole blood and "stopping solution" was removed from the ice bath, dried, and reweighed within 3 to 5 min of its being withdrawn from the animal. Next, the contents of the syringe were gently delivered into a test tube whereupon it was centrifuged (3000 rpm × 10 min at 4°C). Following centrifugation, the plasma was aspirated off and flash frozen for subsequent processing and analysis (see below).

In order to (i) document the ability of "stopping solution" to preserve a known amount of [^{3}H]adenosine in plasma and (ii) determine if recovery of adenosine were influenced by the addition of EDTA to "stopping solution" or use of PCA, additional experiments were performed as follows. A known quantity of tritiated adenosine was added to a test tube containing either "stopping solution" or "stopping solution" plus EDTA or PCA. Blood was then withdrawn into heparinized syringes containing the different "stopping solutions" (but equal amounts of tritiated adenosine) and subsequently processed as described above. Processed plasma samples (or neutralized whole blood extract in the case of PCA) were then subjected to HPLC (vide infra) with collection of appropriate fractions which were subsequently counted for tritium activity. Expected concentration of tritiated adenosine was determined based upon the measured plasma volume and known amount of radioactivity added to the sample. Percentage recovery was determined by comparing observed with expected concentration of tritiated adenosine after accounting for all dilutions involved.

Tritium activity in each sample was determined with a Searle Mark III (Model 6880) liquid scintillation counter. Tritium standards with known activity and quench characteristics were counted for each run. The machine used an external Ba-133 standard along with information contained in the standard quench curve to automatically determine counting efficiency for each sample. Output from the counter included both cpm and dpm as well as percentage counting efficiency. Since all samples exhibited counting efficiencies which varied within a very narrow range (approximately 42–48%), results were analyzed only in terms of cpm.

2. Determination of plasma adenosine concentration. Samples of plasma were first deproteinized by ultrafiltration. This was accomplished by placing the sample in a 25,000 mol wt cut-off ultrafiltration membrane cone (Amicon Co., Danvers, MA, Model CF 25) and centrifuging for 20 min at 1000g and 4°C. Adenosine content of the ultrafiltrate was determined by high-pressure liquid chromatography. The method employed was a modified version of one previously reported by one of us (10). Briefly, 100 μl was injected on a 15-cm long, 5-μ particle size, C-18, VMA reverse-phase column (BioRad Corp., Richmond, CA). A computer-controlled dual pump system was used to pump progressively increasing

concentrations of methanol (60:40 Vol:Vol mixture in distilled water) through the column. The buffer employed was 0.02 *M* KH_2PO_4, pH 5.5. Flow rate was 2.0 ml/min with a linear gradient of 0 to 14% methanol over 15 min. The ultraviolet absorbance (262 nm) of eluent was monitored continuously (range = 0.005 absorbance units, full scale) and recorded on chart paper (10 mV = 25 cm).

Concentration of endogenous adenosine in each sample was determined by comparing observed peak height with that of known standards. Peak identity was determined by retention time and confirmed by (i) disappearance after pretreatment with adenosine deaminase and (ii) augmentation by coinjection with exogenous adenosine. The detection system responded linearly over a range of 2 to 100 pmole; quantities of adenosine < 2 pmole/100 μl injection were not detectable.

3. Determination of plasma clearance of [^{3}H]adenosine. We determined the rate of clearance of [^{3}H]adenosine from porcine blood in the following manner. Tritiated adenosine (approximately 2×10^6 cpm; specific activity = 2.3×10^4 cpm/pmole) dissolved in 0.05 ml of normal saline was added to each of 10 preweighed test tubes. One tube also contained 0.5 ml "stopping solution," another contained 0.5 ml "stopping solution" plus EDTA, while a third contained PCA (0.5 ml). Blood (20 ml) was withdrawn from the animal into a heparinized syringe and 3 ml was added to each of 5 test tubes. The first contained "stopping solution" or "stopping solution" plus EDTA or PCA while the remainder contained only [^{3}H]adenosine. Subsequently, "stopping solution" or "stopping solution" plus EDTA or PCA was added to the remaining test tubes after delays of 0.5, 1, 5, and 20 min. Thus one sample of each sequence had its [^{3}H]adenosine protected at the time of addition of heparinized whole blood while in the remaining samples, [^{3}H]adenosine was exposed to blood for 0.5 to 20 min prior to addition of "stopping solution" (either alone or with EDTA) or PCA. Test tubes were incubated in a rack at room temperature (~20°C) during the study. Blood used for the experiment was kept in a water bath at 37°C while not in use. The pH of blood in the syringe was measured at the start and end of one sequence (duration of 20–30 min) and did not change appreciably (start = 7.44; end = 7.45).

Blood samples were processed as described above and then subjected to HPLC for determination of [^{3}H]adenosine activity. The latter was accomplished by collecting and then counting precisely timed fractions which previously had been determined to coincide with elution of adenosine from the column. Adenosine cpm/ml plasma measured in samples obtained following delayed administration of "stopping solution" or PCA were compared with adenosine cpm/ml plasma in the samples obtained with "stopping solution" present at the time of addition of blood to the sample. Finally, the concentration of unlabeled adenosine in each of these samples also was measured and corrected for added labeled adenosine.

4. Red cell adenosine content and in vitro production of adenosine. In one set of experiments, four 3-ml samples of blood were obtained in heparinized syringes in rapid succession from a single animal. Two of the samples were placed in stopping solution alone and two in stopping solution plus EDTA. One sample with "stopping solution" and one with stopping solution plus EDTA was processed as described previously. Each of the two other samples was subjected to vigorous mechanical hemolysis by placing a small spatula in the tube and vortexing for 5 min. Each of these samples was subsequently centrifuged and then processed in the same fashion as the nonhemolyzed samples.

In order to determine red cell content of adenosine, paired 3-ml samples of blood were obtained in heparinized syringes containing either stopping solution plus EDTA or an equivalent volume of 8% perchloric acid. The acid precipitated sample was subsequently centrifuged and the supernate was decanted off and neutralized after which adenosine concentration was determined by HPLC. Concentrations were corrected for dilution resulting from addition of a small quantity (200 μl) of strong base (4.5 *M* K_2CO_3). The sample obtained in the stopping solution plus EDTA was centrifuged, and the plasma was removed for subsequent determination of adenosine concentration. The red cells from this sample were washed twice with normal saline and then

resuspended in distilled water in order to hemolyze the cells. The hemolysate was spun to remove red cell ghosts, ultrafiltrated as described above, and subsequently analyzed for adenosine content.

Results. *1. Recovery of [^{3}H]adenosine. a. "Stopping solution."* Ten samples of blood were obtained from 10 different animals for this part of the study. An average of 82.0 ± 1.9% (±1 SEM) of [^{3}H]adenosine added to each sample was recovered.

b. "Stopping solution" plus EDTA. Three samples of blood were obtained from three different animals. Recovery of [^{3}H]adenosine was very consistent among the animals (85.9, 84.1, and 88.9%) and also was nearly identical to that observed in matched samples obtained in stopping solution alone (82.0, 86.7, and 87.5%, respectively).

c. PCA. Recovery studies ($N = 3$) with PCA were conducted with blood from three different animals (the same ones described in section 1b above). Values obtained were consistent from animal to animal (70.8, 68.4, and 66.6%, respectively) but were approximately 20% lower than those observed with either "stopping solution" (see 1b, above). Accordingly, values of unlabeled adenosine in PCA-treated samples were multiplied by 1.25 to account for reduced recovery vis-à-vis "stopping solution"-treated samples (correction applies to Tables Ib and II).

2. Clearance of [^{3}H]adenosine from plasma (Table IA, B; Fig. 1). Results with "stopping solution" alone, "stopping solution" plus EDTA and PCA did not differ and were consistent among the three trials (blood from three different animals) which were performed. The results of a representative experiment are shown in Fig. 1. In the absence of "stopping solution" of any kind, contact with whole blood for only 30 sec resulted in loss of 99% of the labeled nucleoside from the plasma. Results for delays of 1 and 5 min were similar (i.e., loss of approximately 99% of the labeled nucleoside). A 20-min delay resulted in loss of 100% of the labeled nucleoside from the plasma.

The concentration of unlabeled adenosine in each of the plasma samples exhibited a consistent and reproducible pattern (Fig. 1). The adenosine (unlabeled) concentration of samples obtained in "stopping solution" alone was always 5- to 10-fold greater than that of matched samples obtained in "stopping solution" plus EDTA. The adenosine (unlabeled) levels in samples obtained after delayed addition of "stopping solution," "stopping solution" plus EDTA, or PCA were generally similar to levels measured in matched samples in which "stopping solution," "stopping solution" plus EDTA, or PCA was present prior to addition of whole blood. Increasing the time delay between collection of the blood sample and addition of any "stopping solution" did not result in a consistent, progressive decline in the unlabeled adenosine concentration of the sample.

TABLE I. TIME DELAY BETWEEN SAMPLING AND ADDITION OF "STOPPING SOLUTION"

	Time delay (minutes)				
	0	0.5	1	5	20
(A) Plasma [3H]Adenosine activity (cpm/0.1 ml; mean ± 1 SEM: $N = 3$)					
SS	28668 ± 3825	309 ± 14	242 ± 43	173 ± 26	0[a]
SS + EDTA	28552 ± 2652	393 ± 62	244 ± 25	208 ± 16	0[a]
PCA	39531 ± 9511	404 ± 79	404 ± 88	231 ± 74	0[a]
(B) Plasma adenosine concentration (nM; mean ± 1 SEM, $N = 3$)					
SS	1036 ± 163[b]	1022 ± 79	934 ± 93	1149 ± 82	1200 ± 144[a]
SS + EDTA	209 ± 84[b]	240 ± 79	156 ± 24	157 ± 28	134 ± 24[a]
PCA	226 ± 52[b]	178 ± 48	171 ± 38	235 ± 84	184 ± 56[a]

Note. Abbreviations used: SS, "stopping solution," PCA, perchloric acid. Values for these samples are those of the neutralized supernate which is *not* the same as plasma.

[a] $N = 2$.

[b] All values corrected for [^{3}H]adenosine added to sample.

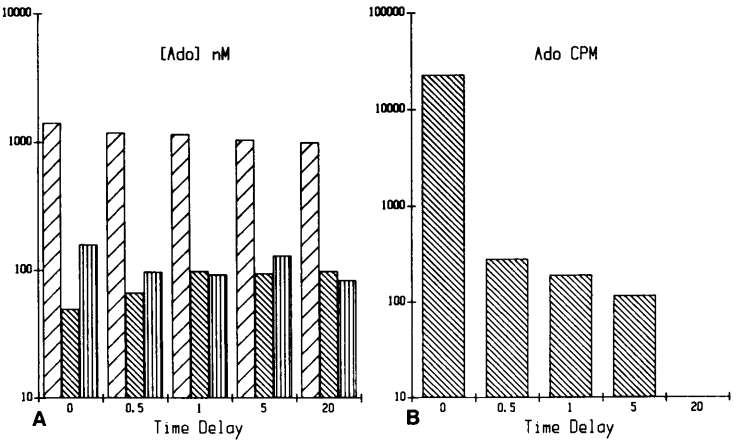

FIG. 1. Results of one experiment are shown here. (A) Plasma adenosine concentration (log scale) on the ordinate versus time delay (minutes) between collection of the sample and addition either of "stopping solution" (▨), or "stopping solution" plus EDTA (▧). Total adenosine content of a matched PCA (▥) sample also is shown. Note that in comparison with delay = 0, plasma (or whole blood) adenosine concentration changes little even with delays as long as 20 min. In addition, plasma adenosine concentration in samples obtained in "stopping solution" alone is roughly 10-fold greater than that of matched samples obtained in "stopping solution" plus EDTA. (B) [^{3}H]Adenosine placed in the test tube was removed almost entirely from plasma after just 30 sec exposure to whole blood. A delay of 20 min resulted in complete loss of [^{3}H]adenosine from plasma. See text for discussion.

3. Red cell adenosine content and production (Tables II and III). a. Adenosine content (Table II). Samples of blood from four different animals were employed for these studies. The animal's hematocrits were essentially the same (mean = 34, range 32–36). The adenosine (endogenous) content of 3 ml whole blood was 311 ± 56 (mean ± 1 SEM) pmole in acid-precipitated samples. The plasma endogenous adenosine content was 80 ± 13 pmole while the red cell content was 157 ± 14 pmole in matched samples of whole blood obtained in "stopping solution" plus EDTA. The endogenous adenosine concentration in the plasma averaged 40 ± 7 n*M* while the red cell concentration (based on total red cell volume in the sample) averaged 153 ± 11 n*M*. Finally, the total endogenous adenosine in the PCA samples (average = 311 pmoles) agreed well with the sum of endogenous adenosine in plasma (average = 80) plus red cell fraction (average = 157) of samples obtained in "stopping solution" + EDTA (i.e., 80 + 157 = 237).

b. Adenosine production (Table III). Me-

TABLE II. RED BLOOD CELL AND PLASMA ADENOSINE CONTENT AND CONCENTRATION (MEAN ± 1 SEM, $N = 4$)

PCA, Blood	Content (pmole/3 ml blood)			Concentration (n*M*), SS + EDTA	
	SS + EDTA				
	Plasma	RBCs	Total[a]	Plasma	RBCs[b]
311 ± 56	80 ± 13	157 ± 14	236 ± 24	40 ± 7	153 ± 11

Note. Abbreviations used: SS, "stopping solution," PCA, perchloric acid.

[a] Sum of plasma and RBC content of 3-ml sample obtained in "stopping solution" (SS) + EDTA (e.g., 51 + 154 = 205).

[b] Red blood cell (RBC) concentration calculated by dividing RBC adenosine content by volume of RBCs in 3-ml sample of blood.

TABLE III. PLASMA ADENOSINE CONCENTRATION (n*M*; MEAN ± SEM) IN PAIRED BLOOD SAMPLES PROCESSED WITH AND WITHOUT HEMOLYSIS

	Hemolysis	
	Absent	Present
SS ($N = 3$)	1388 ± 176	14,144 ± 1349
SS + EDTA ($N = 7$)	78 ± 20	221 ± 40

Note. Abbreviation used: SS, "stopping solution."

chanical hemolysis of whole blood obtained in "stopping solution" alone resulted in substantial production of adenosine. In contrast, hemolysis of matched samples obtained in "stopping solution" plus EDTA did not cause appreciable production of adenosine. In a separate group of studies in which plasma adenosine concentration in nonhemolyzed samples was compared with matched samples subjected to mechanical hemolysis only in "stopping solution" plus EDTA, there was evidence of red cell adenosine production in only one of four trials. In this one case (Experiment No. 0709), the amount of adenosine observed in the hemolyzed sample was more than what could be accounted for by simple release of stored adenosine from the red cells although it was much less than that observed in samples mechanically hemolyzed in "stopping solution" alone.

Discussion. The results of the present study may be summarized as follows. First, removal of [^{3}H]adenosine added to porcine plasma was rapid. Indeed, more than 95% of labeled adenosine was removed from plasma within 20 sec of its addition to the blood. Second, substantial *in vitro* accumulation of adenosine took place when samples of blood were obtained in "stopping solution" alone. This was demonstrated by the fact that blood samples obtained in "stopping solution" alone exhibited adenosine concentrations several fold greater than matched samples obtained in "stopping solution" plus EDTA. The fact that deliberate mechanical hemolysis of blood samples obtained in "stopping solution" alone resulted in plasma adenosine concentrations which were 10-fold greater than those of matched nonhemolyzed samples (Table III) further illustrates the potential for red cells to produce adenosine and for it to accumulate in plasma under *in vitro* conditions in which nucleoside transport is blocked and adenosine deaminase is inhibited. While leukocytes and platelets also may have contributed to *in vitro* production of adenosine, the fact that erythrocytes are so much more numerous in blood favors a predominant role for the red cell in this regard. Accordingly, since substantial *in vitro* production of adenosine takes place in samples of blood obtained in "stopping solution" alone, it is apparent that such samples are unlikely to be useful for measuring uptake or production of adenosine by organs of the body such as the heart.

Enzymes such as (Ca^{2+}, Mg^{2+}) ATPase and 5′-nucleotidase bound on cell membranes or contained within the cytosol of formed elements of blood require divalent cations for activity (11, 12). Continued activity of these enzymes during sample processing coupled with inhibition of nucleoside transport (dipyridamole) and adenosine deamination (EHNA) likely is responsible for *in vitro* accumulation of adenosine. In addition, even very minor trauma to erythrocytes during processing may cause nucleotides to leak from the cell and subsequently be converted to adenosine by ectonucleotidases (13). Addition of EDTA to "stopping solution" results in chelation of divalent cations and inactivation of these same enzymes. Accordingly, *in vitro* accumulation of adenosine should be greatly reduced, if not eliminated entirely, by addition of EDTA to "stopping solution." The data obtained in this study (Tables IB and II) clearly support this hypothesis. The fact that samples of blood obtained in PCA exhibited total adenosine content similar to the combined plasma plus red cell content of matched samples obtained in "stopping solution" plus EDTA also supports the hypothesis that ongoing activity of red cell enzyme systems (i.e., Ca^{2+}, Mg^{2+} ATPase, 5′-nucleotidase) in conjunction with inhibition of nucleoside transport and deamination is largely responsible for *in vitro* accumulation of adenosine in blood samples obtained in "stopping solution" alone.

Although addition of EDTA to "stopping solution" will minimize *in vitro* accumulation of adenosine, it will not influence the rapidity

with which adenosine is cleared from plasma. Furthermore, even though labeled adenosine is rapidly cleared from plasma in the absence of "stopping solution," plasma adenosine concentration of blood collected in "stopping solution" plus EDTA is very similar to that of matched samples in which a delay of up to 20 min has elapsed between the time of collection and addition of "stopping solution" with EDTA (Table IB). In the absence of "stopping solution" constant plasma concentration of the nucleoside over time despite rapid loss of [^{3}H]adenosine from plasma suggests the hypothesis that formed elements of the blood, particularly erythrocytes, may contribute importantly to regulation of plasma adenosine concentration. It would appear that adenosine added to blood is rapidly cleared by red cells and then is subsequently released back into the plasma (presumably after cycling through one or more metabolic pools) at a reduced rate. The fact that there was no tritiated adenosine in plasma samples in which a delay of 20 min had elapsed between the time of collection and addition of "stopping solution" plus EDTA, despite the fact that the concentration of unlabeled adenosine was unchanged versus matched samples placed immediately in "stopping solution" plus EDTA supports this hypothesis. In essence, the data are consistent with buffering of plasma adenosine by red cells (and possibly leukocytes and platelets) through removal of added adenosine and release of nucleoside from the cell. The observation that red cell adenosine concentration is roughly three- to fourfold greater than that of plasma (Table II) also supports this hypothesis and is consistent with earlier observations made by Mills *et al.* (14). Finally, it is unlikely that adenosine measured in plasma of samples obtained with a delay between collection and addition of "stopping solution" plus EDTA is the result of *in vitro* production since all but three values observed were less than those measured in matched samples obtained in PCA.

Rapid removal of tracer adenosine from plasma observed in this study deserves further comment. Experiments by previous investigators have demonstrated considerable variability in this regard. In two studies rapid removal of adenosine from canine plasma (20–65% within 10 sec) was reported (15, 16) while in two other canine experiments very much slower removal was observed (roughly 16%/min (6) and 20%/min (8)). The data obtained in the present study (>95% removal within 10 sec) are more consistent with results of Olsson *et al.* (15) and Rubio *et al.* (16). Furthermore, in a recent study of adenosine transport kinetics in human erythrocytes it was shown that K_m and V_{max} for this process were on the order of 5 μM and 50 μmole/liter/min, respectively (2). Accordingly, the first-order rate consant (k) for the process in humans is roughly 10 min^{-1} ($k = V_{max}/K_m$) and the half-time is approximately 0.07 min ($t_{1/2} = 0.693/k$). An identical first-order rate constant for adenosine removal from rabbit blood can be calculated from the data of Catravas (17). Thus, in human or rabbit blood nucleoside transport alone would permit more than 95% of adenosine added to plasma to be taken up by red cells within 20 sec, almost precisely the result observed in the present study. We recognize, however, that all mammalian erythrocytes do not possess identical adenosine transport systems (18). Nevertheless, the data obtained in our study are consistent with results of human (2), rabbit (17), and some canine (15, 16) studies which indicate that removal of adenosine added to plasma of these species occurs very rapidly.

In conclusion, the objective of this study was to test the hypothesis that plasma adenosine concentration of blood samples obtained in conventional "stopping solution" are falsely elevated. The data obtained clearly demonstrate that (i) the hypothesis is correct and (ii) ongoing *in vitro* production of adenosine in association with inhibition of nucleoside transport (dipyridamole) and deamination (EHNA) plays an important role and leads to substantial accumulation of adenosine in the sample. Finally, rapid uptake of adenosine added to plasma in species with active erythrocyte nucleoside transport systems, and the tendency for intraerythrocytic and plasma adenosine pools to maintain equilibrium suggests the need for caution in employing measurements of plasma adenosine concentration to assess the role of the nucleoside in physiological processes such as myocardial blood flow regulation.

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