Disposition of Intravenously Administered Adenosine 5'-Phosphosulfate (APS) in Rats

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The disposition of adenosine 5'-phosphosulfate (APS), an endogenous nucleotide, was investigated in rats. The degradation of APS in rat plasma was very rapid. APS was degraded in rat plasma to AMP and ATP, and these nucleotides were further degraded through adenosine. The degradation kinetics was examined. For the *in vivo* study, the method to protect APS from degradation in blood was examined, and it was found that the addition of EDTA to APS-containing blood and storage at 4°C can protect against APS degradation. After intravenous bolus injection, APS in plasma declined rapidly and the rate of elimination was dose-dependent: the biological half-life was about 2 s at the dose of 0.3 mg/kg and was longer at 3 mg/kg. When APS was administered by intravenous infusion, the plasma level rapidly reached a steady-state, which then rapidly declined after the infusion was stopped. The total body clearance of APS could not be fully explained by metabolism in plasma or glomerular filtration, therefore the contribution of other elimination processes to the total body clearance was suggested.

Key words adenosine 5'-phosphosulfate; plasma degradation; kinetic analysis; APS; rat

Inorganic sulfate is activated by the reaction of two enzymes. In the first step of the activation, ATP accepts the inorganic sulfate and is then converted to adenosine 5'-phosphosulfate (APS).¹⁾ These activating enzymes were found in Furth mouse mast cell,²⁾ rat chondrosarcoma,³⁾ rat liver,⁴⁾ rabbit liver and calf brain.⁵⁾ Recently, the kinetic mechanism of sulfate activation in rat chondrosarcoma was reported by Lyle *et al.*^{6,7)}

The sulfation of drugs or endogenous substances is one of the most common conjugation reactions in the body. In this case, a sulfate is given by sulfotransferase from the activated form phosphoadenosinephosphosulfate (PAPS), which has been synthesized by APS-phosphokinase from APS and ATP. APS in biological tissues is degraded by enzymes to adenosine (ADO) through AMP.^{8,9)} Although it is well known that APS is a precursor of PAPS, its fate in biological tissues and its biological activity has not been studied in detail.

In this study, we examined the method of analysis of APS in plasma and blood, the kinetic analysis of APS degradation in rat plasma *in vitro* and the pharmacokinetics of APS in rats *in vivo*.

MATERIALS AND METHODS

Materials APS sodium salt (M.W. 449.3) was purchased from Sigma Chemical Co., St. Louis, MO. All other chemicals were reagent grade commercial products and were used without further purification. A vacuum tube (VP-100H, with heparin sodium salt; VP-FH052, with heparin sodium salt, sodium fluoride and EDTA disodium salt) was purchased from Terumo, Tokyo, Japan.

Measurement of APS Metabolism in Rat Plasma Whole blood was collected with a heparinized syringe from male Wistar rats weighing 250—300 g. Blood was centrifuged at 3000 rpm for 10 min at 4°C and the plasma was used immediately. Three hundred μ l of saline solution containing APS was added to 2.7 ml of plasma

preincubated for 5 min at 37°C, and the mixture was incubated at 37°C. The reaction was stopped by deproteinization of the plasma with twice the volume of methanol, followed by centrifugation at 10000 rpm for 1 min. APS and its metabolites in the supernatant fraction were simultaneously assayed by HPLC. The degradation rates of metabolites of APS in plasma were similarly measured using each metabolite as the substrate.

The effect of EDTA on the degradation of APS in rat plasma was examined by the addition of EDTA at a final concentration of 10 μ g/ml. The EDTA-added plasma was incubated at 37°C or stored in a refrigerator (4°C). The reaction was stopped by deproteinization with methanol, and the remaining APS was assayed as described above.

The degradation of APS in blood was examined by incubating APS-spiked blood at 37°C. The reaction was stopped by the addition of EDTA at a final concentration of 10 μ g/ml, and then the blood was centrifuged at 10000 rpm for 1 min. The APS remaining in the supernatant fraction was assayed as described above. The initial concentrations in plasma were calculated using a hematocrit value of 44.3%.

Intravenous Administration of APS Male Wistar rats (200—300 g) were anesthetized with urethane and were fixed on an operation plate. The right femoral artery and left femoral vein were cannulated with vinyl tubing. For the bolus injection study, APS saline solution was administered into the femoral vein at a dose of 0.3, 1 or 3 mg/kg. About 100 μ l of blood sample was collected from the cannulated vinyl tube on the right artery by auto-spouting (for exactly 5 s) and quickly mixed with an anticoagulant, which is a mixed powder of heparin sodium salt, sodium fluoride and EDTA disodium salt in a VP-FH052 vacuum tube.

For the constant infusion study, APS saline solution was infused into the femoral vein for 30 min at the rate of 0.476 or 1.0 mg/kg/min. Each sample was centrifuged at 10000 rpm for 1 min at room temperature. Forty μ l of

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Fig. 1. Typical HPLC Chromatograms

(A) Sample of a mixture of APS, ADO, AMP, ADP and ATP in rat plasma (10 µg/ml each). (B) Sample of APS in rat blood (2.5 µg/ml). Peaks: 1=APS; 2=ADO; 3=AMP; 4=ADP; 5=ATP.

the supernatant (plasma) was mixed with 80 µl of methanol, and APS in the supernatant after the second centrifugation (10000 rpm for 1 min) was assayed by HPLC.

In some experiments, constant intravenous infusion was carried out after an intravenous bolus injection of probenecid (20 µmol/kg) or in renal vasculature-ligated rats. The renal vasculature ligation was performed as described in our previous paper.¹⁰⁾

Assay Procedures The supernatant of deproteinized plasma was filtered through a 0.45 µm pore-sized filter (Nihon Millipore Kogyo, Yonezawa, Japan). An aliquot of the filtrate was injected to HPLC. An HPLC pump (LC-6A, Shimadzu, Kyoto, Japan) was equipped with a UV detector (SPD-2A, Shimadzu), a column oven (CTO-2A, Shimadzu) and an integrator (CR-3A, Shimadzu). The system was operated in a reversed phase with a TSK-Gel ODS-80TM column (4.6 × 150 mm, Toso Co., Tokyo). Ion-pair chromatography was performed using a mobile phase of 10 mM KH₂PO₄: methanol containing 1 mM tetra-*n*-butylammonium perchlorate (87:13 by volume). The flow rate was 1.0 ml/min and the column temperature was kept at 40 °C. The wavelength was 254 nm. APS and the metabolites, ATP, AMP and ADO, in plasma could be determined simultaneously under this condition (Fig. 1A), but the metabolites were hard to determine from the blood sample (Fig. 1B).

The pyrophosphate (PP_i) concentration in rat plasma was measured using a Phospha C Test Wako (Wako Pure Chemical Industries, Osaka, Japan) after incubation at 37 °C for 30 min with 3.3 U of pyrophosphatase (Unitika, Ltd.).

Data Analysis The parameters for the degradation in plasma were determined from differential equations by the non-linear least squares fitting program "MULTI (RUNGE)".¹¹⁾

RESULTS AND DISCUSSION

Metabolic Fate of APS in Rat Plasma As described in Assay Procedures, APS and the metabolites, ATP, AMP and ADO, in rat plasma could be determined simultaneously by our simple HPLC method. The retention times

Fig. 2. Concentration Dependent Degradation Profiles of APS in Rat Plasma

Initial concentrations of APS were 20, 50 and 115 nmol/ml. Results are expressed as the mean ± S.E. of 3 experiments.

of APS, ADO, AMP, ADP and ATP were 13.7, 2.0, 3.2, 10.3 and 47.8 min, and the coefficients of variation (C.V.) values were 2.7, 6.8, 4.9, 2.4 and 3.9% ($n=8$), respectively, in plasma. The detection limits for APS and the metabolites were 0.1–0.2 µg/ml.

The metabolism of APS in rat plasma was examined at 3 different concentrations (20, 50 and 115 nmol/ml) and the result is shown in Fig. 2. The concentration of endogenous APS in the blank plasma was below the detection limit (<0.1 µg/ml) by our assay method. As is evident from the figure, the degradation was rapid: the degradation half-lives are 1.1, 1.7 and 2.9 min at the initial concentrations of 20, 50 and 115 nmol/ml, respectively. The elimination process is non-linear.

Figure 3 shows the time courses of plasma concentrations of the metabolites AMP, ADO and ATP following the incubation of APS in rat plasma. Initial concentrations of APS were 14, 35 and 70 nmol/ml. These nucleotides were further metabolized through ADO. APS has been considered to be enzymatically degraded to ADO through AMP (Fig. 4A).^{8,9)} However, the disposition parameters of AMP and ADO (K_m , V_{max} and k_1) estimated simultaneously from Fig. 3 and following 3 differential equations were different from our previous data¹²⁾ (Table

Fig. 3. Time Courses of APS and Its Metabolites during APS Degradation in Rat Plasma

Initial concentrations of APS were 70 (A), 35 (B) and 14 (C) nmol/ml. Results are expressed as the mean $\pm$ S.E. of 3 experiments. $\bullet$, APS; $\circ$, ATP; $\triangle$, AMP; $\square$, ADO.

Table 1. Kinetic Parameters of APS Degradation Process in Rat Plasma

Process in Fig. 4		K_m (nmol/ml)	V_{max} (nmol/min/ml)	k_1 (min^{-1})
(A)	1	59.8	29.9	—
	4	10.3	13.2	—
	5	—	—	0.0858
(B)	1	3.2 ± 2.8	4.0 ± 0.4	—
	2	8.9 ± 6.6	3.5 ± 0.6	—
	3	$10.7 \pm 6.0^a)$	$12.2 \pm 4.4^a)$	—
	4	$22.3 \pm 4.6^a)$	$14.2 \pm 0.8^a)$	—
	5	—	—	$0.0450 \pm 0.003^a)$

a) Values from reference 12).

1 (A) vs. (B)), which were estimated individually from the disposition of AMP and ADO.

$$\frac{d[\text{APS}]}{dt} = -\frac{V_{\max_1}[\text{APS}]}{K_{m_1} + [\text{APS}]} \quad (1)$$

$$\frac{d[\text{AMP}]}{dt} = \frac{V_{\max_1}[\text{APS}]}{K_{m_1} + [\text{APS}]} - \frac{V_{\max_4}[\text{AMP}]}{K_{m_4} + [\text{AMP}]} \quad (2)$$

$$\frac{d[\text{ADO}]}{dt} = \frac{V_{\max_4}[\text{AMP}]}{K_{m_4} + [\text{AMP}]} - k_1[\text{ADO}] \quad (3)$$

where K_{m_i} and $V_{\max_i}$ are the Michaelis constant and maximum velocity of process i , respectively, and k_1 is the first-order degradation rate constant of ADO.

This means that Fig. 4A is not sufficient to explain the metabolic fate of APS in rat plasma. Thus, other pathways of APS degradation seem to exist. From the information provided by HPLC analysis, a part of APS was degraded to ATP in rat plasma (Fig. 3). For ATP production from APS, pyrophosphate (PPi) is needed. The concentration of PPi in rat plasma was 0.40 ± 0.00 mM (mean $\pm$ S.E., $n=3$), while the APS concentration in the reaction was about 0.2 mM. Therefore, this PPi concentration is sufficient to prepare ATP from APS. ATP is degraded to AMP by ATP pyrophosphohydrolase in plasma, but not to ADP, since ATPase is an ectoenzyme on the surface of blood cells or on the cells of the vascular lining.¹³⁾ In addition, ADP was not detected in plasma during the APS degradation. Thus, ADP is not included in the APS degradation pathway in rat plasma. A new model for APS disposition in rat plasma was proposed (Fig. 4B) in which

Fig. 4. Two Possible APS Disposition Pathways in Rat Plasma

APS is degraded to ATP or AMP, then ATP to AMP, AMP to ADO, and ADO to non-detected materials, probably inosine. The differential equations for this new model were considered as follows:

$$\frac{d[\text{APS}]}{dt} = -\frac{V_{\max_1}[\text{APS}]}{K_{m_1} + [\text{APS}]} - \frac{V_{\max_2}[\text{APS}]}{K_{m_2} + [\text{APS}]} \quad (4)$$

$$\frac{d[\text{ATP}]}{dt} = \frac{V_{\max_2}[\text{APS}]}{K_{m_2} + [\text{APS}]} - \frac{V_{\max_3}[\text{ATP}]}{K_{m_3} + [\text{ATP}]} \quad (5)$$

$$\frac{d[\text{AMP}]}{dt} = \frac{V_{\max_1}[\text{APS}]}{K_{m_1} + [\text{APS}]} + \frac{V_{\max_3}[\text{ATP}]}{K_{m_3} + [\text{ATP}]} - \frac{V_{\max_4}[\text{AMP}]}{K_{m_4} + [\text{AMP}]} \quad (6)$$

$$\frac{d[\text{ADO}]}{dt} = \frac{V_{\max_4}[\text{AMP}]}{K_{m_4} + [\text{AMP}]} - k_1[\text{ADO}] \quad (7)$$

Using Figs. 2 and 3 and differential Eqs. 4—6, degradation parameters for processes 1 and 2 were determined. The metabolic pathways of processes 3—5 in Fig. 4B have been examined in rat plasma individually, using ATP, AMP and ADO as substrates.¹²⁾ The kinetic constants estimated individually are summarized in Table 1, and simulation lines based on the model shown in Fig. 4B and the kinetic constants of each process listed in Table 1 are shown in Fig. 3. The lines fit relatively well with the experimental data, suggesting that the model of Fig. 4B accurately explains the metabolic fate of APS in rat plasma.

Protection of APS from Degradation in Rat Blood In order to determine APS concentration in a blood sample, blood cells must be removed from the blood sample before the analysis of the plasma APS. At least 5 min is needed to complete the deproteinization of plasma. However, most of the APS in the blood sample is degraded during the process. Thus, APS must be protected from degradation in the blood. Since the enzyme degrading APS was thought to need divalent cations, such as Mg^{2+} and

Ca^{2+} , as in the case of diadenosine 5',5'''- P^1P^4 -tetraphosphate (Ap4A)-degrading enzyme,¹⁴⁾ EDTA is expected to inhibit the degradation by chelating divalent cations in rat blood. The stability of APS in rat plasma with EDTA (10 $\mu\text{g}/\text{ml}$) is shown in Fig. 5. APS degraded

Fig. 5. Effect of EDTA on APS Degradation in Rat Plasma

Initial concentration of APS was 10 $\mu\text{g}/\text{ml}$. Results are expressed as the mean $\pm$ S.E. of 3 experiments. $\bullet$, plasma denatured with methanol; Δ , untreated plasma at 37°C; $\blacktriangle$, untreated plasma at 4°C; $\square$, plasma with EDTA at 37°C; $\blacksquare$, plasma with EDTA at 4°C.

Fig. 6. Plasma Concentration-Time Curves of APS after Intravenous Bolus Injection in Rats

Doses of APS were 0.3 ($\circ$), 1.0 (Δ) and 3.0 ($\square$) mg/kg. Results are expressed as the mean $\pm$ S.E. of 3 experiments.

slowly in rat plasma at 37°C in the presence of EDTA (the half-life was 5.35 h), but APS in EDTA-added plasma was stable when stored in refrigerator (4°C). Under this condition, APS remained completely intact for 7 h. From these results, analysis of APS concentration can be performed by treating the blood sample with EDTA and storing it in a refrigerator. However, EDTA mixed with blood sample interferes the analysis of AMP and ADO by HPLC.

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Figure 6 shows the plasma concentration-time curves of APS after intravenous bolus administration at doses of 0.3, 1.0 and 3.0 mg/kg. The elimination of APS was very rapid at all of the doses, and the biological half-life estimated from the terminal phase was 2.0 s. Since the saturable metabolism in plasma and the urinary excretion by glomerular filtration should be included in the elimination process of APS *in vivo*, a one-compartment model with parallel saturable and first-order elimination processes was applied. Thus, the plasma concentration of APS (C_p) can be expressed by the following equation.

$$\frac{dC_p}{dt} = -\frac{V_{\max}C_p}{K_m + C_p} - k_{el}C_p \quad (8)$$

where K_m and $V_{\max}$ are the pooled parameters of the Michaelis constant and the maximum velocity of the saturable elimination processes, respectively, and k_{el} is a first-order elimination rate constant.

Figure 7 shows the plasma concentration-time curves of APS after intravenous constant infusion at rates of 0.476 and 1.0 mg/kg/min. The plasma concentrations each reached steady state in a short time due to the very short biological half-life, and declined very rapidly by stopping the infusion. The steady-state levels (C_p^{ss}) were 1.03 ± 0.09 and 2.57 ± 0.04 $\mu\text{g}/\text{ml}$ at the infusion rates of 0.476 and 1.0 mg/kg/min, respectively, and were nearly proportional to the rate at these 2 doses. The total body clearance (CL_{total}) was calculated to be 425.8 ml/min/kg from the steady state levels in Fig. 7. Since the disappearance of plasma APS after stopping the infusion was very rapid, it was hard to estimate the apparent elimination rate constant. Thus, from the slopes of the terminal phase of the plasma concentration-time curves in Fig. 6, the value

Fig. 7. Plasma Concentration-Time Curves of APS after Intravenous Constant Infusion in Rats

Infusion rates of APS were 0.476 ($\circ$) and 1.0 (Δ) mg/kg/min. Infusions were stopped at 15 min. Lines (C_p) were drawn by the following equations. (A) For $0 < t < 15$ min, $C_p = C_p^{ss}(1 - \exp(-20.4t))$; (B) For $t > 15$ min, $C_p = C_p^{ss}\exp(-20.4t)$, where C_p^{ss} values are 1.03 and 2.57 $\mu\text{g}/\text{ml}$ at the infusion rates of 0.476 and 1.0 mg/kg/min, respectively. Results are expressed as the mean $\pm$ S.E. of 3–4 experiments.

of the apparent first-order elimination rate constant ($V_{\max}/K_m + k_{el}$ in case of Eq. 8) was estimated to be 20.4 min^{-1} . From these values, the apparent volume of distribution (V_d) could be calculated as 20.9 ml/kg . This value is slightly smaller than the plasma volume, suggesting the property of poor extravascular distribution. From this V_d value, the plasma concentrations at time 0 (C_p^0) in Fig. 6 are calculated to be 14.4, 47.9 and $143.7 \mu\text{g/ml}$ for 0.3, 1.0 and 3.0 mg/kg , respectively. The plasma concentration–time data in Fig. 6 could not be well simulated when the K_m and $V_{\max}$ values were used simply as K_{m1} , K_{m2} , $V_{\max1}$ and $V_{\max2}$ in Table 1, respectively: these parameters can explain only 8% of the terminal slopes of the plasma concentration–time curves. The presence of a capacity-limited elimination process other than metabolism in plasma is suggested.

Next, the data in Fig. 6 were simultaneously fitted to Eq. 8, and K_m , $V_{\max}$ and k_{el} values were estimated. However, the k_{el} value was very small and could be neglected from the Akaike's information criterion (AIC) values. Since the glomerular filtration rate in rats ($4\text{--}12.5^{15}$) or 5.24^{16} ml/min/kg) explain less than 3% of the terminal slopes of the data in Fig. 6, this exclusion of k_{el} from Eq. 8 is reasonable. As a consequence, the plasma concentration–time data of APS can be expressed as Eq. 9, similarly to the case of Ap4A.¹²⁾

$$\frac{dC_p}{dt} = -\frac{V_{\max}C_p}{K_m + C_p} \quad (9)$$

The lines in Fig. 6 are simulation lines by Eq. 9, where the K_m and $V_{\max}$ values were $6.25 \pm 0.37 \mu\text{g/ml}$ and $17.21 \pm 2.02 \mu\text{g/s/ml}$, respectively. These K_m and $V_{\max}$ values are the pooled parameters of parallel Michaelis-Menten equations and are much larger than those for the metabolism in plasma, suggesting the presence of other capacity-limited elimination processes with low affinity and high capacity.

In order to clarify the contribution of the kidney to APS elimination, the CL_{total} was examined in renal vasculature-ligated (a kind of nephrectomy) rats by the constant infusion. In these rats, both renal arteries and veins were ligated after laparotomy. As shown in Table 2, the CL_{total} was markedly reduced in the renal vasculature-ligated rats, suggesting a significant role of the kidney in APS elimination *in vivo*, and the renal clearance can be calculated as 203.4 ml/min/kg . This is much greater than the renal blood flow (32.1^{15}) or 36.8^{16} ml/min/kg). This might result from a reduction of the metabolic activity in other organs in the renal failure state. Since rats pretreated with probenecid ($20 \mu\text{mol/kg}$, i.v.)¹⁷⁾ showed no change in the CL_{total} , the contribution of an active tubular secretion by the anion transport mechanism cannot be clarified.

The clearance of APS metabolism in plasma is predicted to be 51.2 ml/min/kg with a value of 31.2 ml/kg as plasma volume.¹⁵⁾ The sum of the renal clearance of APS (203.4 ml/min/kg) and the metabolism of APS in plasma (51.2 ml/min/kg) can not explain the CL_{total} of APS (389.1 ml/min/kg). However, the kinetic parameters of APS degradation in plasma determined by *in vitro* experiments with or without blood cells were similar (Fig.

Table 2. Role of Kidney in Total Body Clearance of APS in Rats

Treatment	CL_{total} (ml/min/kg)
None	389.1 ± 5.5 (4)
Pretreatment with probenecid ^{a)}	388.5 ± 53.4 (3)
Renal vasculature-ligation	185.7 ± 22.1 (4) ^{b)}

The CL_{total} values of APS were calculated from the steady-state plasma concentration following intravenous infusion at the rate of 1.0 mg/kg/min . Results are expressed as the mean $\pm$ S.E. with the number of experiments in parentheses. a) The infusion was started 3 min after the intravenous bolus injection of probenecid ($20 \mu\text{mol/kg}$). b) $p < 0.001$.

Fig. 8. Degradation Profiles of APS in Rat Blood

Initial concentrations of APS in blood were 14.4 (Δ) and 43.1 ($\bullet$) nmol/ml . Open symbols are degradation profiles in plasma at the concentrations of 14 (Δ) and 35 ($\circ$) nmol/ml . Lines were drawn using Eq. 4, where K_{m1} , K_{m2} , $V_{\max1}$ and $V_{\max2}$ are the values shown in Table 1(B). Results are expressed as the mean $\pm$ S.E. of 3–4 experiments.

8). It is possible that other processes of APS degradation, such as pulmonary metabolism,¹⁸⁾ in rats exist. The details of these processes remain to be clarified.

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