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PROPERTIES AND SUB-CELLULAR DISTRIBUTION OF TWO SULFATASES WHICH DEGRADE ADENOSINE 5'-PHOSPHOSULFATE

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SUMMARY

Adenosine 5'-phosphosulfate (APS)-sulfatase splits sulfate from APS, the direct precursor of 3'-phosphoadenosine 5'-phosphosulfate (PAPS) "active sulfate". The properties and sub-cellular distribution of APS-sulfatase activity are described in pig kidney cortex. The evidence is consistent with the existence of two species of APS-sulfatases. One enzyme species has an acid and the other a neutral pH optimum. The two differ in their electrophoretic distribution patterns. Activities are concentrated in the vesicles of the smooth endoplasmic reticulum (smooth-4 fraction), in lysosomes, and in the nuclear fraction. K_m values indicate a high affinity of these enzymes for APS. APS-sulfatases were also studied in the human and in the rat. The position of these APS-sulfatases in the pathways of sulfate metabolism suggests that they may play an important role in governing sulfate conjugation from PAPS.

INTRODUCTION

3'-Phosphoadenosine 5'-phosphosulfate (PAPS; active sulfate) serves as the sulfate donor in many important biochemical reactions. ROBBINS AND LIPMANN[1] showed that adenosine 5'-phosphosulfate (APS) is the immediate precursor of PAPS. Hence, enzyme systems which regulate the concentration of APS would seem to play an important biological role in governing sulfate conjugation. Evidence consistent with APS-sulfatase activity was noted by SPENCER[2] who found that APS was completely hydrolysed by rat liver.

The purposes of the present report are: (a) to describe some properties of two sulfatases which catabolize APS to AMP and SO_4^{2-}; (b) to note their different sub-cellular distributions, and (c) to comment on some possible physiological implications of these findings.

Abbreviations: APS, adenosine 5'-phosphosulfate; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; PAP, 3'-phosphoadenosine 5'-phosphate; SAP, adenosine (2' or 3')-sulfate 5'-phosphate; S_2AP, adenosine 2',3'-disulfate 5'-phosphate; S_3AP, adenosine 2',3'-disulfate 5'-phosphosulfate; SA, adenosine (2' or 3') sulfate, PHMB, *p*-hydroxymercuribenzoate.

METHODS

Sub-cellular fractionation. Fresh pig kidney was fractionated with sucrose solutions by methods identical to those specified in a previous report from this laboratory[3].

Preparation of supernatants. Supernatants were prepared from whole adult pig kidney cortex, from whole human kidney and brain, and from rat kidney cortex, liver, and brain. The human material was obtained at autopsy from a 3-year-old boy who died suddenly of acute trauma to the cervical spine. The human samples were frozen at −20° for 5 months before assay. Rat tissues were obtained from a young adult. All samples were homogenized in distilled water (2 ml/g) with a Duall homogenizer, centrifuged at 13 000 × *g* for 30 min, decanted and dialysed overnight in H_2O at 5°. After a final centrifugation at 13 000 × *g* for 15 min, the supernatant was routinely used for assay, save for the exceptions noted.

Preparation of PAPS and APS. Carrier-free [^{35}S]PAPS was obtained from rat liver[4] using the partially purified PAPS synthesizing system[5]. [^{35}S]APS was synthesized according to REICHARD AND RINGERTZ[6] with the following modifications: the reaction mixture contained 0.5 g ATP, 0.25 ml pyridine, 3 mC (carrier free) $H_2{}^{35}SO_4$ in 1.0 ml water, 0.075 ml conc. H_2SO_4, and 2.5 g dicyclohexylcarbodiimide. At 2 and 4 h, dicyclohexylcarbodiimide (2.5 g) was added; at 5.5 h, the reaction was stopped with 50 ml water and extracted 6 times with 50-ml aliquots of water. The water extracts were then extracted with 100 ml of ether (× 4). The final water solution was concentrated (by lyophilization) to 75 ml, passed upward through a 2 cm × 16 cm Dowex 50W-X8 column, and the [^{35}S]APS was eluted with 250 ml cold (4°) water. 3 g of activated charcoal were then added, stirred for 0.5 h at 0–4°, and centrifuged at 15 000 × *g* for 10 min. The charcoal was washed twice with 100 ml cold water and recentrifuged each time. [^{35}S]APS was eluted from the charcoal with 75 ml ethanol–0.4% NH_3 (1:1, v/v). This was repeated 3 times. The solution was concentrated to 100 ml *in vacuo* and centrifuged at 25 000 × *g* for 10 min to remove more charcoal. This final solution containing the [^{35}S]APS was filtered through a type HA Swinney millipore filter, concentrated to dryness and electrophoresed on Whatman No. 1 filter paper at 400 V for 2 h in 0.05 M NH_3–ammonium acetate buffer (pH 9.5). The following bands were identified by their characteristic positions on autoradiographs, following which they were eluted and counted: adenosine [^{35}S](2′ or 3′)-sulfate (SA) (2.3 cm/h), adenosine 5′-phosphosulfate (APS) 2.7 cm/h. APS (2.7 cm/h), adenosine (2′ or 3′)-sulfate 5′-phosphate (SAP) (3.8 cm/h), adenosine 2′,3′-disulfate 5′-phosphosulfate (S_3AP) (4.2 cm/h), and $SO_4{}^{2-}$ (6.6 cm/h), all ^{35}S-labelled. Although the method of REICHARD AND RINGERTZ[6] does not give the highest possible yield of [^{35}S]APS, it does provide [^{35}S]SAP as a valuable by-product. [^{35}S]-APS remained stable for at least 6 months when frozen at pH 8. The two preparations used in this study had specific activities of 0.95 and 1.68 mC/mmole. Other nucleotides were obtained commercially.

Assay system for APS-sulfatase activity. The incubation mixture contained 0.1 ml of enzyme solution and 0.1 ml of 0.4 M Tris–acetate buffer at either pH 5.0 or pH 7.0. To this was added 0.02–0.04 ml [^{35}S]APS (5 000–10 000 counts/min or 1.9–3.8 nmoles). The mixture was incubated at 37° for 30 min in a Lab-Line, Model 2095 shaker. After incubation, 0.56 ml of 0.1 M K_2SO_4 was added. The incubation mixture was thereafter treated as previously specified. APS-sulfatase activity is

expressed as nmoles of substrate hydrolysed per ml enzyme per h. The amount of free $^{35}SO_4^{2-}$ in the various [^{35}S]APS preparations averaged 2.3%. The appropriate blank value was routinely subtracted from the above.

The non-radioactive assay system contained 0.05 ml APS (5 mM), 0.05 ml enzyme solution and 0.1 ml of pH 5 or pH 7 buffer. After incubation for 15 min at 37° in the shaker, the reaction was stopped with 0.3 ml acetone and centrifuged twice at 4000 × *g* for 10 min to remove the charcoal. 15 μl of the clear supernatant were applied to Beckman S+S No. 2043 A filter paper, and electrophoresed for 2 h at 400 V at pH 6.65. The buffer consisted of equal parts of 0.05 M citrate–phosphate (pH 6.65) and 0.1 M Tris–acetate (pH 6.65). The paper was viewed under ultraviolet light, and areas corresponding to the APS and AMP standards were cut out, eluted with distilled water and read at 260 mμ on a Beckman DU spectrophotometer. Other sulfatases were assayed as previously described, PAPS-sulfatase[3], sulfatase A (ref. 7), and sulfatase B (ref. 7).

Electrophoretic demonstration of APS-sulfatase activity. Aliquots (0.03 ml) from the various supernatants were applied in bands across the middle of strips of Beckman S+S No. 2043 A filter paper. The aliquots contained the following amounts of protein: 0.64 mg (pig kidney); 0.63 mg (rat kidney); 1.58 mg (rat liver); and 0.15 mg (rat brain). The strips were equilibrated with 0.05 M barbiturate buffer (pH 8.5) and electrophoresed at 200 V for 16 h (4°). The strips were next dried with a fan for 2 min and cut lengthwise. Each duplicate strip was dipped into an ^{35}S-labelled APS solution at pH 5 or pH 7. Calculations indicated that each strip (measuring 1.5 cm × 30 cm) contained approx. 112 000 counts of ^{35}S-labelled APS. The strips were incubated for

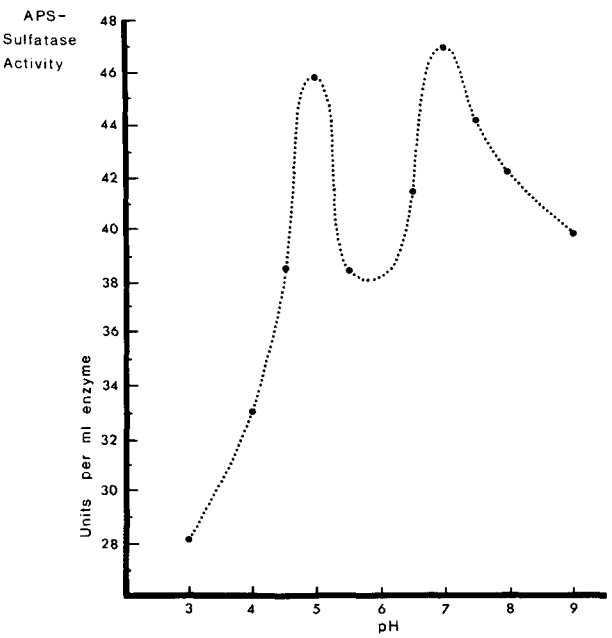

Fig. 1. Dual pH optima of the APS-sulfatase system in pig kidney supernatant. Assay conditions are described in the text. 1 ml enzyme solution contained 12.7 mg protein. 1 unit = $4.8 \cdot 10^{-1}$ nmoles [^{35}S]APS hydrolysed.

TABLE I

THE EFFECT OF COBALT* ON ADENOSINE 5'-PHOSPHOSULFATE SULFATASE ACTIVITY (PIG KIDNEY SUPERNATANT)

Sample	*Activity (nmoles/ml enzyme per h)*	*Change (%)*
pH 5**	21.1	
pH 5 + Co^{2+}	21.9	4
pH 6**	10.8	
pH 6 + Co^{2+}	11.6	7
pH 7**	31.6	
pH 7 + Co^{2+}	32.1	2

* Cobalt was taken up in 0.4 M Tris-acetate buffer (pH 5.0, 6.0, or 7.0) so that 0.1 ml contained the correct amount for a final concentration of 5 mM. Assay system as described in the text.

** Assayed in separate experiments.

30 min at 37° in a water saturated tank and dried at room temperature. The $^{35}SO_4^{2-}$ locally released was precipitated by immersing each strip for 3 min in a saturated solution of barium acetate. Unhydrolysed [^{35}S]APS was then removed by soaking the strip in two changes of distilled water for 1 h with agitation every 5 min. For radioautography, the strips were then exposed to Chronex 2 DC film for 21 days.

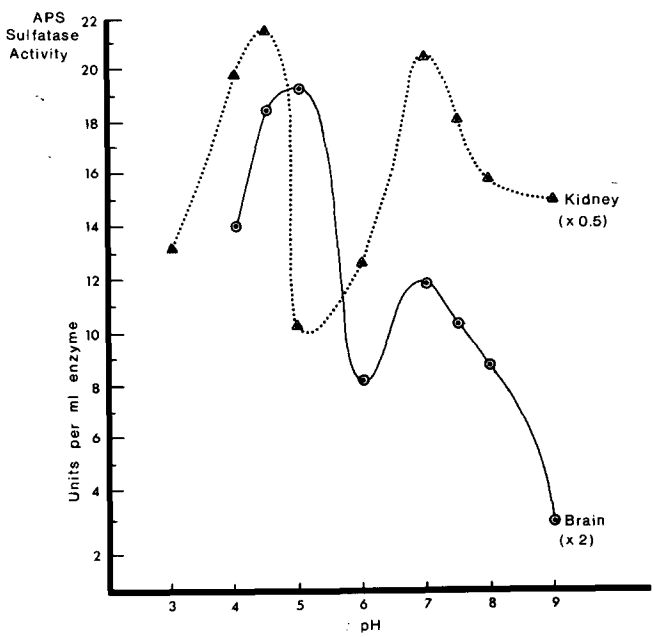

Fig. 2. Dual pH optima of the APS-sulfatase system in human brain and kidney supernatants. The brain sample was 90% cerebral cortex and 10% underlying white matter. The kidney sample was 50% cortex, 50% medulla. Assay conditions are described in the text. Kidney values are $^1/_2$ of the original activity and brain values twice the original activity. One ml enzyme solution contained 10.8 mg protein (kidney) and 6.7 mg of protein (brain). 1 unit = $4.8 \cdot 10^{-1}$ nmoles [^{35}S]APS hydrolysed.

RESULTS

pH optima of the two APS-sulfatases in tissue supernatants

Two pH optima are seen (pH 5 and pH 7) when APS-sulfatase activity is assayed in the 13 000 × *g* supernatant obtained from a distilled water homogenate of unfractionated pig kidney tissue (Fig. 1). Cobalt had no appreciable effect on the activity seen at either pH optima or at pH 6 (Table I). In contrast, PAPS-sulfatase activity was highest at pH 6.0 and was stimulated (26%) by 0.01 M cobalt.

Supernatants from human kidney and brain also show two pH optima (Fig. 2). Human kidney and pig kidney had approximately the same amount of sulfatase activity at either pH, but human brain had 39% more activity at pH 5. Added cobalt did not stimulate the activity in human brain or kidney.

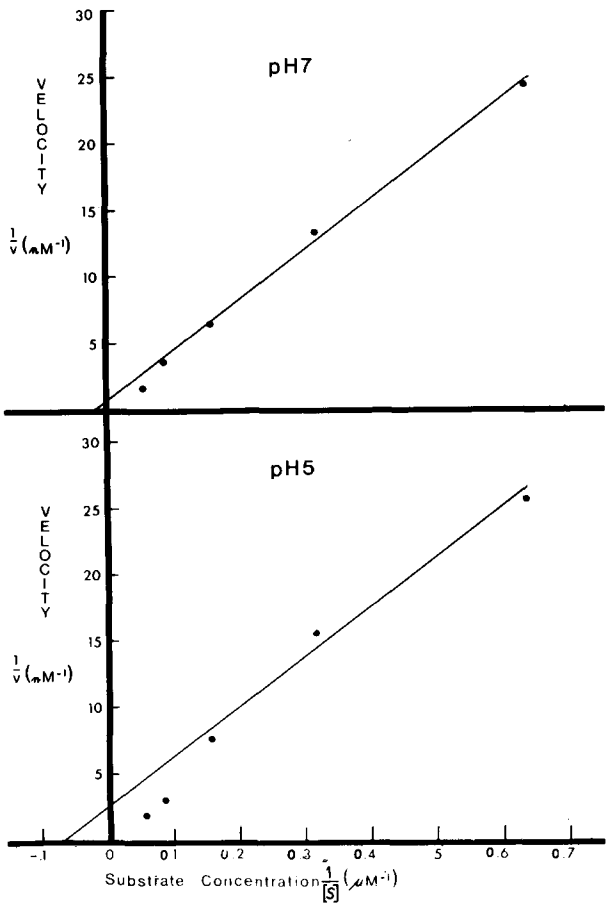

Fig. 3. The effect of varying substrate concentration on the APS-sulfatase activity of pig kidney. The protein concentration was 17.7 mg/ml. The *r* value was calculated for the activity at pH 5. A value of 0.993 was found for APS concentrations between $1.59 \cdot 10^{-6}$ and $6.34 \cdot 10^{-6}$ M. Values for *r* this close to 1.000 imply that there is an essentially linear relationship between substrate concentration and enzyme velocity. The *r* value for APS-sulfatase activity at pH 7 was 0.999 (for APS concentrations between $1.59 \cdot 10^{-6}$ and $11.61 \cdot 10^{-6}$ M).

The effect of varying substrate concentrations of APS-sulfatase activity

From the Lineweaver–Burk plot (Fig. 3) K_m values can be calculated for the sulfatase activities at each pH. The K_m at pH 5 is $15.6 \cdot 10^{-6}$ M, and that at pH 7 is $68.8 \cdot 10^{-6}$ M.

The effect of varying enzyme concentration on enzyme activity

At pH 5, enzyme activity, at first, increased when the enzyme solution was diluted up to 10-fold (Fig. 4). Indeed, the activity at 40 times dilution was similar to the original activity. Even at 1000-fold dilutions, the enzyme at pH 5 retained 34%

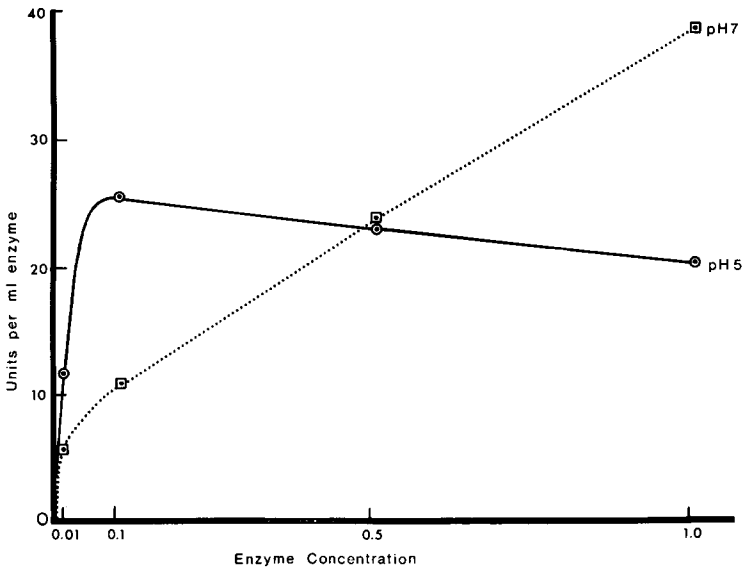

Fig. 4. The effect of varying enzyme concentration on the APS-sulfatase activity of pig kidney. Each curve represents the mean of two sets of determinations. The protein concentrations were 17.7 and 18.8 mg/ml in the original 13 000 × *g* supernatant. These solutions were further diluted with buffer to give the lower concentrations indicated (dilution factors shown are 2, 10, and 100.) The shapes of these curves were reproducible.

of its original activity. By contrast, the activity at pH 7 continually decreased with dilution, and at 1000 times dilution, it retained 10% of its original activity.

The relationship between APS-sulfatase activity and the activities of enzymes which degrade PAPS and SAP

The results in Table II indicate that neither PAPS nor SAP is a substrate for the APS enzymes, under the conditions of the experiment.

Stability of the enzymes

After homogenization and dialysis, the supernatant was stored at 0° for varying lengths of time. The activities present thereafter are presented in Table III. The activity at pH 7 appeared more stable than that at pH 5.

TABLE II

THE RELATIONSHIP BETWEEN APS-SULFATASE ACTIVITIES AND THE ACTIVITIES OF ENZYMES WHICH DEGRADE PAPS AND SAP (PIG KIDNEY SUPERNATANTS)

Substrate	*Initial counts*	*Counts of $^{35}SO_4^{2-}$ released at pH 5*	*Hydrolysis (%)*	*Counts of $^{35}SO_4^{2-}$ released at pH 7*	*Hydrolysis*
[^{35}S]APS	5460 (2.62 nmoles)	4978 (2.39 nmoles)	91	5284 (2.54 nmoles)	97
[^{35}S]SAP	1707 (0.82 nmoles)	90 (0.04 nmoles)	5	87 (0.04 nmoles)	5
[^{35}S]PAPS	2542* (1.04 nmoles)	0	0	0	0

* Assay system as described in the text. [^{35}S]SAP and [^{35}S]PAPS were added as a volume aliquot (0.04 ml and 0.001 ml, respectively) to the incubation mixture.

TABLE III

STABILITY OF PAPS-SULFATASE ACTIVITY FROM PIG KIDNEY

Assay system as described in the text. The routine supernatant (13 000 × *g*) was allowed to stand at 0° and then re-assayed at the indicated times.

Duration of storage at 0° (h)	*Activity at pH 5 (nmoles/ml enzyme per h)*	*Change from original (%)*	*Activity pH 7 (nmoles/ml enzyme per h)*	*Change from original (%)*
24	22.6	0	22.6	0
48	18.7	−17	22.6	0
72	17.3	−23	20.6	−9

TABLE IV

THE EFFECT OF VARIOUS IONS ON APS-SULFATASE ACTIVITY IN PIG KIDNEY SUPERNATANT

Assay system as described in the text. The various substances were taken up in 0.4 M Tris–acetate buffer at either pH 5.0 or pH 7.0, so that 0.1 ml contained the correct amount for final concentrations indicated.

Substance	*Concn. (mM)*	*Activity at pH 5 (nmoles/ml enzyme per h)*	*Change from original (%)*	*Activity at pH 7 (nmoles/ml enzyme per h)*	*Change from original (%)*
None		22.1	0	22.6	0
F^-	13	22.1	0	18.2	−19
F^-	50	18.2	−18	32.2	30
F^-	100	20.2	−9	27.8	19
PO_4^{3-}	5	16.3	−26	17.8	−12
SO_3^{2-}	25	21.6	−2	27.4	18
Mg^{2+}	10	17.3	−22	16.8	−26
Co^{2+}	5	23.0	4	23.0	2
EDTA	10	19.2	−14	20.2	−11
Glutathione*	10	15.4	−30	19.2	−15
Iodoacetate	10	19.2	−14	25.0	10

* Reduced form.

The contrasting effects of various ions on APS-sulfatase activity

The results in Table IV indicate that both activities are inhibited to approximately the same extent by PO_4^{3-}, Mg^{2+}, and EDTA. Although Mg^{2+} and Co^{2+} are necessary for 5'-nucleotidase and PAPS-sulfatase activity, neither cation stimulated APS-sulfatase activity. The enzyme activity at pH 7 was stimulated by: SO_3^{2-}, iodoacetate and F^- (at 50 and 100 mM). It is of note, however, that these same ions inhibit the sulfatase activity assayed at pH 5. Pre-incubation of the enzyme with buffer only for 15 min at 37° caused a slight decrease in the activity at pH 5 (6%) and a slight increase in that at pH 7 (12%).

Radioautography of electrophoretically separated APS-sulfatase activities

The two activities differ considerably; the pH 5 activity was, in general, more cathodal and the pH 7 activity was more anodal. Thus, at pH 5 there were two major active bands in pig kidney and rat kidney. One was present at the application point, the other migrated 1.5 cm toward the cathode. Pig kidney also had two additional

TABLE V

THE EFFECT OF PHMB ON APS-SULFATASE ACTIVITY IN PIG KIDNEY AND RAT TISSUE SUPERNATANTS

The assay system consisted of 0.1 ml enzyme, $+2.5 \cdot 10^{-4}$ M PHMB in 0.05 ml of 0.05 M glycine–NaOH buffer (pH 8.0). After 1 h pre-incubation at 38° in a shaker, 0.05 ml of 0.8 M Tris–acetate buffer at pH 5.0 or pH 7.0, and 4500 counts/min (1.22 nmoles) of [^{35}S]APS was added and incubated for 30 min. The remainder of the assay system was as described in the text.

Sample (species and organ)	*Activity at pH 5 (nmoles/ml enzyme per h)*	*Activity at pH 5 $+2.5 \cdot 10^{-4}$ M PHMB (nmoles/ml enzyme per h)*	*Change (%)*	*Activity at pH 7 (nmoles/ml enzyme per h)*	*Activity at pH 7 $+2.5 \cdot 10^{-4}$ M PHMB (nmoles/ml enzyme per h)*	*Change (%)*
Pig kidney cortex	11.3	13.2	+15	14.3	16.5	13
Rat kidney cortex	13.5	10.0	−26	17.3	20.0	15
Rat liver	11.1	6.2	−44	17.0	17.0	0
Rat brain*	20.5	23.5	+13	23.8	8.4	−65

* Whole brain was used .The composition was estimated at 60% cerebral cortex and 40% underlying white matter. Cerebellum wat not included in this sample.

minor bands which moved toward the anode (1.5 and 3.5 cm). Rat kidney did not show these minor bands. Rat liver showed one major band at the application point and one that moved 0.7 cm toward the cathode. Rat brain showed no activity under these conditions.

At pH 7, pig kidney, rat kidney, and rat liver all showed a major band at the application point. In addition, rat kidney showed a broad, dense band of activity extending 3 cm from the application point toward the anode. Rat kidney also showed a minor anodal band at 6 cm. Rat liver showed 2 minor anodal bands at 5 and 8.5 cm. Rat brain had 3 faint anodal bands (2.5, 6.0, and 8.5 cm).

The effect of PHMB on APS-sulfatase activity

Pre-incubation of the pig supernatants with $2.5 \cdot 10^{-4}$ M *p*-hydroxymercuribenzoate (PHMB) showed no inhibition of the pH 5 or pH 7 activities. However, rat brain, liver, and kidney activities did show varying inhibition with PHMB (Table V), both at pH 5 and pH 7.

The contrasting effects of various nucleotides on APS-sulfatase activity

These are summarized in Table VI. Low concentrations of AMP and ADP inhibit the activity at pH 7 more than that at pH. 5. However, at higher ADP concentrations (50 mM), the activity at pH 5 is inhibited to a greater degree.

SAP also affects the two activities quite differently. SAP has virtually no effect on the pH 5 enzyme, but it inhibits the activity at pH 7 to an appreciable degree (−32%). PAP (at the same concentration) caused a slight increase in both activities.

TABLE VI

THE EFFECT OF VARIOUS NUCLEOTIDES ON APS-SULFATASE ACTIVITY IN PIG KIDNEY IN PIG KIDNEY SUPERNATANT

Assay system as described in the text. The various nucleotides were taken up in 0.04 M Tris–acetate buffer at either pH 5.0 or pH 7.0, so that 0.1 ml contained the correct amount for the final concentrations indicated.

Substance added	*Concn. (mM)*	*Activity at pH 5 (nmoles/ml enzyme per h)*	*Change from original (%)*	*Activity at pH 7 (nmoles/ml enzyme per h)*	*Change from original (%)*
None		22.1		22.6	
AMP	5	19.7	−11	16.3	−28
ADP	5	20.6	− 7	17.3	−24
ADP	25	12.5	−44	18.2	−19
ADP	50	5.8	−74	11.0	−51
ATP	5	19.2	−14	25.0	−10
ATP	25	3.4	−85	3.8	−83
[^{35}S]PAPS	$1 \cdot 10^{-6}$	16.8	−24	20.6	− 9
PAP	0.001	24.0	8	26.4	14
PAP	5	2.4	−89	28.8	22
[^{35}S]SAP	$0.8 \cdot 10^{-6}$	22.6	2	15.4	−32

Higher PAP concentrations severely inhibited the activity at pH 5 (−89%), but stimulated the activity at pH 7 (+22%). [^{35}S]PAPS inhibited the activity at pH 5 more than that at pH 7.

The effect of high-speed centrifugation on the distribution of two APS-sulfatase activities

Supernatants routinely used for the studies cited above were obtained after centrifuging at 13 000 × *g*. In the experiments now to be described, this supernatant was further centrifuged at 105 000 × *g* for 60 min. This yielded a pellet fraction containing microsomes and other membranous elements, and a clear high speed supernatant. The results are summarized in Table VII.

Removal of membranous components slightly increased the activity found in the residual (high-speed) supernatant both at pH 5 and 7. Activity was also present

TABLE VII

THE EFFECT OF HIGH SPEED CENTRIFUGATION ON THE DISTRIBUTION OF THE TWO APS-SULFATASE ACTIVITIES FROM PIG KIDNEY

Assay system as described in the text. An aliquot of the routine supernatant was centrifuged at 105 000 × *g* 60 min to yield the high-speed supernatant and a pellet composed of microsomes and membranes. The microsomal-membrane pellet was re-suspended in a minimum volume of double distilled water and assayed.

Sample assayed	*Activity at pH 5 (nmoles/ml enzyme per h)*	*Activity at pH 7 (nmoles/ml enzyme per h)*
Routine supernatant (13 000 × *g*)	22.1	22.6
High-speed supernatant *minus* membranes (105 000 × *g*)	24.5	26.4
High-speed microsomal-membrane pellet	4.8	20.2

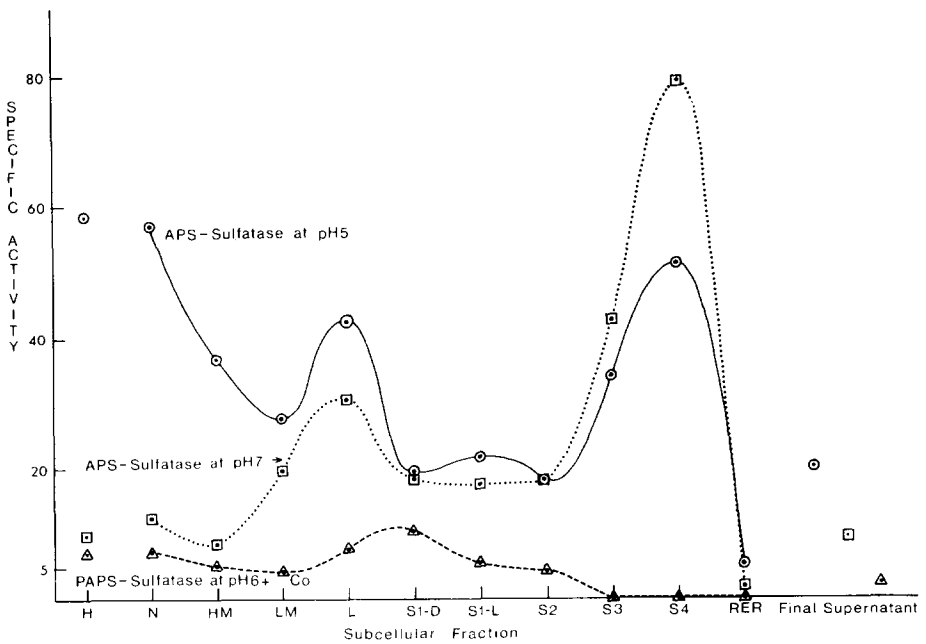

Fig. 5. The sub-cellular distribution of APS-sulfatase activities at pH 5 and pH 7 (as described in the text) and of PAPS-sulfatase activity at pH 6 *plus* Co^{2+} (ref. 4). The abbreviations refer to the following fractions: H = original homogenate; N = nuclear; HM = heavy mitochondrial; LM = light mitochondrial; L = lysosomal; S-1 (D) = smooth 1 dense; S-1 (L) = smooth 1 light; S-2 = smooth 2; S-3 = smooth 3; S-4 = smooth 4; RER = rough endoplasmic reticulum. Note that the PAPS-sulfatase activities should be multiplied by 20 to reach their original value. Activities still remaining in the final high-speed supernatant are indicated at the right. One specific activity unit is defined as the hydrolysis of 1000 counts/min ($2.7 \cdot 10^{-1}$ nmoles) of [^{35}S]APS, and as the hydrolysis of 1000 counts/min ($4.1 \cdot 10^{-1}$ nmoles) of [^{35}S]PAPS per mg protein per h.

in the pellets, particularly the activity of the APS-sulfatase assayed at pH 7. Indeed, the sum of the high-speed supernatant and pellet activities assayed at pH 7 was twice that of the original routine supernatant from which they were derived (96.2 *vs.* 46.9, respectively). The above findings suggested the possibility that the two APS-sulfatase activities might be referable to somewhat different sub-cellular loci.

Sub-cellular distribution patterns

APS-sulfatase activity at pH 5 is chiefly referable to the lysosomal fraction and the smooth-4 fraction of the endoplasmic reticulum (Fig. 5). APS-sulfatase activity at pH 7 is also chiefly referable to the same two fractions. However, their proportional relationships differ. In comparison with the lysosomal fraction, there is only 17% more of the pH 5 activity in the smooth-4 fraction, whereas there is 39% more of the pH 7 activity in this same smooth-4 fraction. In the nuclear fraction, the level of pH 5 activity is considerably higher than that of pH 7 activity, both in absolute terms and in relation to that of other sub-cellular fractions.

For contrast, PAPS-sulfatase activity is chiefly concentrated in the lysosomal and S-1 (D) fractions and is lacking in the smooth-3 through rough endoplasmic reticulum fractions.

All three activities were low in the final high-speed supernatant obtained after all organelles were removed. Regarding levels found in the original homogenate as 100%, the levels in the final supernatants were: APS-sulfatase (pH 5), 8.9%; APS-sulfatase (pH 7), 24.1%; and PAPS-sulfatase, 7.4%.

TABLE VIII

$(NH_4)_2SO_4$ FRACTIONATION OF 13 000 × *g* SUPERNATANT OF PIG KIDNEY CORTEX

The routine supernatant described in the text was not dialyzed but immediately fractionated by adding increasing amounts of $(NH_4)_2SO_4$, stirring for 15 min and then centrifuging at 10 000 × *g* for 15 min to obtain the respective pellets. Each pellet was re-suspended in a minimum volume and dialyzed overnight in H_2O at 5°. The final solutions were assayed directly for the various enzyme activities.

$(NH_4)_2SO_4$ (M)	*Enzyme activity for each $(NH_4)_2SO_4$ fraction (%)*					*Adenosine†† from 5'-AMP via 5'-APS at pH 5*
	*APS sulfatase at pH 5**	*APS sulfatase at pH 7**	*PAPS-sulfatase***	*Aryl-sulfatase A****	*Aryl-sulfatase B†*	
0–0.34	4	4	9	2	3	0
0.34–0.67	25	17 (69 nmoles)	28	34	14	0
0.67–1.01	26 (138 nmoles)	11	20	33	1	Trace
1.01–1.34	6	9	13	11	22	1+
1.34–1.68	14	11	9	10	38	2+
1.68–2.02	9	13	9	6	13	3+
2.02–2.35	7	13	8	3	5	4+
2.35–3.36	13	22	5	1	3	0

* Assay system as described in the text. The amount of 5'-AMP released was read at 260 nm in comparison with a standard curve of 5'-AMP.

** PAPS-sulfatase[3] assay system contained 55 547 counts/min (22.77 nmoles).

*** Arylsulfatase A (ref. 7).

† Arylsulfatase B (ref. 8).

†† The relative amounts of adenosine were estimated visually under ultraviolet light.

$(NH_4)_2SO_4$ fractionation of the two APS-sulfatase and of other sulfatase activities

In these studies, unlabelled APS was used at higher concentrations (5 mM). The results are summarized in Table VIII. APS-sulfatase activity did not precipitate at any one concentration. There were three peaks in the APS-sulfatase activity assayed at pH 5. These occur at $(NH_4)_2SO_4$ saturations between 0.34 and 1.01 M, 1.34 and 1.68 M; 2.35 and 3.36 M. There were two peaks in the APS-sulfatase activity assayed at pH 7: one between 0.34 and 0.67 M; the second between 2.35 and 3.36 M $(NH_4)_2SO_4$ saturation.

PAPS-sulfatase activity had only one peak occurring between 0.34 and 0.67 M saturation. Arylsulfatases A and B were assayed as control sulfatases and also had different distribution peaks.

AMP is formed during the initial breakdown of APS. The adenosine subsequently released by AMP 5'-phosphatase could be followed by paper electrophoresis. Its peak activity occured at an $(NH_4)_2SO_4$ saturation between 2.02 and 2.35 M. This finding was not evident in the earlier radioautographs of these fractions because the radioactive [^{35}S]APS did not contain sufficient adenosine to be identified under ultraviolet light.

DISCUSSION

Significance of the observation that APS-sulfatase activity has a double pH optima

The present report appears to be the first showing that nucleotide sulfatase activity can have a dual pH optima. The two pH optima occur both in pig kidney, human kidney, and human brain. LEVIN AND BODANSKY[8] found that a 5'-nucleotidase (monophosphatase) present in bull semen also showed this phenomenon. These authors note that several other hydrolase activities also exhibit this same behaviour. There is, however, at least one other 5'-nucleotidase, present in lysosomes, which has but a single pH optimum[9].

SCHWIMMER[10] analyses five general situations which could give rise to double pH optima: (a) two distinct isoenzymes having different pH optima; (b) active enzyme-substrate complexes by two ionic species of the enzymes which differ by at least two protons; (c) the presence of an ampholyte inhibitor, only one ionic species of which can combine with the enzyme; (d) the presence of an ampholyte activator; (e) inhibition by an excess of ampholyte substrate. On the basis of his proposals, our results would seem to suggest that: (1) there are two sulfatases present, each having different characteristics; (2) amphoteric inhibitors or activators may also be present in the supernatant which influence enzyme velocity.

Evidence favouring the existence of two enzymes is that the activities present at pH 5 or pH 7 show clear-cut differences in the following respects: (a) in their response to anions; (b) in their response to nucleotides; (c) in their stability at 0°; (d) in their sub-cellular distribution; (e) in their patterns of precipitation at various concentrations of $(NH_2)_4SO_4$, and (f) in their distribution patterns after electrophoretic separation.

There remains the evidence that certain anions (SO_3^{2-}, and F^-) may stimulate activity at one pH (Table III). It is possible that they do so by blocking specific amphoteric inhibitors. These same ions might inhibit activity at the other pH optima, perhaps by blocking the corresponding amphoteric activator. SO_3^{2-} and F^- inhibit arylsulfatases A and B (lysosomal enzymes with acid pH optima) as well as many

other sulfatases[11]. By contrast, F^- does not affect arylsulfatase C (a microsomal enzyme which has an alkaline pH optima)[12].

It will be noted that PAP was the only nucleotide which stimulated APS-sulfatase activity at pH 7 (Table V). Conceivably, this represents an allosteric effect of PAP. For example, YAMAZAKI AND HAYAISHI[13] reported that ATP caused an allosteric stimulation of nucleoside diphosphatase. They suggested that ATP was bound at a site different from that of the normal substrate. This might stabilize a conformational change in the enzyme causing more activity. Allosteric stimulation is accepted as an important property of a regulatory enzyme[14].

The pH 5 and pH 7 APS-sulfatases differ in their electrophoretic distribution patterns, and also differ from one species (pig) to another (rat). These differences include: net charge, number of bands, and relative amounts of activity present per mg of protein applied.

At the completion of our studies, there appeared a preliminary report in regard to a sulfatase purified from rat liver capable of hydrolysing APS (ref. 15). Unlabelled APS was used as substrate. After being purified 500-fold, this enzyme showed only one pH optimum at 5.2 in acetate buffer. The concentrated activity yielded 6 protein bands on gel electrophoresis. As in the present study, Co^{2+} and Mn^{2+} had no effect upon its activity. ATP, ADP, and pyrophosphate were inhibitory.

PHMB inhibited this APS-sulfatase purified from rat liver[15]. However, in the present study, pig APS-sulfatases were not inhibited by PHMB, either at pH 5 or pH 7. To examine this point, PHMB was added to rat liver, kidney, and brain supernatants ($13\,000 \times g$) prepared as in the pig.

In the rat we found that: (1) the liver APS-sulfatase was inhibited (44%) at pH 5, but not at pH 7; (2) the kidney APS-sulfatase was inhibited (26%) at pH 5, but not at pH 7; and, (3) the brain activity was not inhibited at pH 5, but was (65%) at pH 7. These results suggest that the APS-sulfatases of pig kidney differ from many other sulfatases (which are inhibited by PHMB) and also differ in this respect from those reported in the rat.

The high affinity of the APS-sulfatases for APS

Linear regression analysis (Fig. 3) gave r values of 0.993 and 0.999 at pH 5 and pH 7, respectively[16]. From this plot, the Michaelis constant (K_m) for APS-sulfatase at pH 5 was $15.6 \cdot 10^{-6}$ M and that at pH 7 was $68.8 \cdot 10^{-6}$ M. Thus, the enzymes have a very high affinity for APS—sufficiently high to suggest that APS is, indeed, their "natural substrate". The K_m for purified rat liver APS-sulfatase was $5 \cdot 10^{-4}$ M (ref. 15). ROBBINS AND LIPMANN[1] showed that the synthetic yeast enzyme system which converts APS, namely APS-kinase, also had a very low optimal substrate concentration of only $4.5 \cdot 10^{-6}$ M APS.

For purposes of comparison, the PAPS-sulfatase of sheep brain has a K_m of $4.5 \cdot 10^{-5}$ M (ref. 4), arylsulfatase A has a K_m of $0.8 \cdot 10^{-3}$ M (ref. 17), and arylsulfatase B has a K_m of $6.8 \cdot 10^{-2}$ M (ref. 18). Using a natural substrate (cerebroside sulfate) arylsulfatase A had a K_m of $10.5 \cdot 10^{-5}$ M (ref. 19). Insoluble microsomal sulfatase C (ref. 20) and microsomal steroid sulfatase[12] have K_m values of $2 \cdot 10^{-3}$ and $4 \cdot 10^{-5}$ M, respectively. Thus, in relation to these other sulfatases, both APS-kinase and the APS-sulfatases have extraordinarily high affinities for their substrates. 5'-Nucleotide

phosphatases which have dual pH optima also have K_m values ranging from $8 \cdot 10^{-6}$ to $48 \cdot 10^{-6}$ M (ref. 8).

Even when diluted 1000 times, APS-sulfatase at pH 5 still retains 34% of its original activity, and APS-sulfatase at pH 7 retains 10% of its original activity (Fig. 4). The curves in Fig. 4 again indicate the existence of two enzymes. We have not been able to find in the literature enzyme concentration curves of allosteric enzymes followed over a 1000-fold range for comparison. Full interpretation of the slope of these curves is beyond the scope of the present paper.

Specificity of the sulfatases for APS

There is the possibility that if only one enzyme were present, it might act as a specific sulfatase at one pH, whereas at the other pH it might have a non-specific action. This phenomenon has been alluded to in regard to several phosphatases; carbamyl phosphatase[21], lysosomal acid phosphatase[22], and pyrimidine nucleotide phosphatases[23]. There is no evidence that this phenomenon occurs with respect to PAPS, however, for neither APS-sulfatase hydrolyses PAPS (despite the fact that PAPS, like APS, also contains a 5'-phosphosulfate group). Nor is SAP hydrolysed by either of the two APS sulfatases (Table II). This indicates that these sulfatases show no 3'-sulfatase activity under these experimental conditions.

Effects of certain ions, salts, and other compounds on enzyme activities

Fluoride, above 50 mM inhibits at pH 5, but stimulates at pH 7. This might indicate that some sulfatase inhibitor was being blocked at pH 7. Glutathione and EDTA, known inhibitors of PAPS degradation[2,4,24] was also inhibitory, especially at pH 5. Although cations were not required, EDTA did cause some inhibition. This might mean that prosthetic groups on the enzyme may contain a metal ion which could chelate with EDTA (ref. 9).

PAPS inhibited both sulfatases. Although neither enzyme hydrolyzes PAPS, PAPS might inhibit competitively because it and APS share identical 5'-phosphosulfate groups. It is of note that SAP inhibited at pH 7, but not at pH 5. This result might indicate that each enzyme tends to bind substrate at the 3'- as well as at the 5'-position, but that it is not capable of hydrolysis at the 3'-position. This possibility is raised because both 3'-sulfate and phosphate have similar negative charges.

PAP, in 5 mM concentration, inhibited only the sulfatase activity at pH 5. PAP stimulated the activity at pH 7, both at 1 and 5 mM concentrations. It is of note that two allosteric nucleotide diphosphatases (one soluble, the other microsomal) also show a similar divergent response to ATP (ref. 25).

$(NH_4)_2SO_4$ fractionation revealed no clear-cut areas of maximal APS enzyme concentration, unlike PAPS-sulfatase and arylsulfatase A. For this reason, salt fractionation was not used for isolation purposes, but was used only to indicate that the APS enzymes differ from other control sulfatases.

General significance of these findings

In regard to the possible pivotal biological role of APS as the precursor of PAPS, it is of interest to find that two cytoplasmic enzymes are delegated the role of hydrolysing APS, and that each shows a high affinity for its substrate. In our previous studies it was found that PAPS-phosphatase activity was concentrated in the vesicles

of the smooth-4 fraction[3]. This enzyme system yields APS. Therefore, it is of interest that the highest specific activity of APS-sulfatase is also concentrated in this same smooth-4 fraction. The smooth-4 fraction has no PAPS-sulfatase activity[3]. Thus, it might be proposed: (a) that any excess PAPS in this fraction would be converted back to APS, and (b) that the level of this APS would in turn be regulated by its own sulfatases.

$$\text{PAPS} \xrightarrow{\text{PAPS-phosphatase}} \text{APS} \xrightarrow{\text{APS-sulfatases}} \text{AMP}$$

Other nucleotides present either as beginning or end products of these reactions (AMP, ADP, ATP, and PAP) could also influence the mechanisms governing sulfate conjugation (Table VI). The remainder of the cell, especially lysosomes and contents of the nuclear fraction may also function in the general degradation of APS and PAPS.

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