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ENZYMATIC DEPHOSPHORYLATION OF 3'-PHOSPHOADENOSINE 5'-PHOSPHOSULFATE TO ADENOSINE 5'-PHOSPHOSULFATE IN SHEEP BRAIN

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SUMMARY

An enzyme catalyzing the dephosphorylation of 3'-phosphoadenosine 5'-phospho[^{35}S]sulfate to adenosine 5'-phospho[^{35}S]sulfate was partially purified from sheep brain. The enzyme showed an optimum pH of 5.0, it was activated by EDTA and inhibited by the divalent metal ions tested. ADP and 3'-AMP had no significant influence on the enzyme activity, but 3'-phosphoadenosine 5'-phosphate was a potent inhibitor. NaF completely inhibited the reaction. The enzyme exhibited properties much different from those of 3'-nucleotidase and 3'-phosphoadenosine 5'-phosphosulfate sulfohydrolase of brain. The physiological role of the phosphohydrolase may be in the regulation of the concentration of 3'-phosphoadenosine 5'-phosphosulfate.

INTRODUCTION

3'-Phosphoadenosine 5'-phosphosulfate (PAPS) is recognized to be the biological sulfate donor in the formation of several sulfated compounds[1]. The enzymatic synthesis of PAPS and the transfer of sulfate from PAPS to mucopolysaccharides and sulfatides in brain was demonstrated in earlier work from this laboratory[2–4]. The enzymatic degradation of PAPS to inorganic sulfate was observed in several mammalian tissue extracts[5–8], and the properties of this enzyme, PAPS sulfohydrolase from sheep brain was described earlier[5].

The formation of adenosine 5'-phosphosulfate (APS) as a degradation product of PAPS was reported by SPENCER[6] in rat-liver supernatant and by SUZUKI AND STROMINGER[7] and HARADA *et al.*[9] in hen oviduct preparations. ROBBINS AND LIPMANN[10] used a 3'-nucleotidase (3'-ribonucleotide phosphohydrolase, EC 3.1.3.6) from rye grass for the enzymatic conversion of PAPS to APS. It was observed[11] that free APS as an intermediate in the synthesis of PAPS is not formed in significant quantities especially because of the highly unfavorable equilibrium of the ATP sulfurylase (ATP sulfate adenylyltransferase, EC 2.7.7.4) reaction in the direction of formation

Abbreviations: APS, adenosine 5'-phosphosulfate; PAPS, 3'-phosphoadenosine 5'-phosphosulfate.

of APS and also because of the high affinity of the enzyme APS kinase (ATP adenylylsulfate 3'-phosphotransferase, EC 2 7 1 25) for APS Furthermore it has been shown in this laboratory[12] that APS remains bound to a protein during its enzymatic synthesis by a purified preparation of ATP sulfurylase It would appear that the major route by which free APS is formed is by the enzymatic dephosphorylation of PAPS The present report is concerned with studies on the enzymatic conversion of PAPS to APS catalyzed by a partially purified PAPS phosphohydrolase from sheep brain Evidences are also presented to indicate that the enzyme differs from brain PAPS sulfohydrolase and 3'-nucleotidase in several of its characteristics

MATERIALS AND METHODS

Carrier free $^{35}SO_4^{2-}$ was obtained from Atomic Energy Establishment (Trombay, India) 3'-Phosphoadenosine 5'-phosphate was a gift from Dr J H Austin All other nucleotides were from Sigma Chemical Co (U S A) DEAE-cellulose (Selectacel reagent) and DEAE-Sephadex A-25 were products of Carl Schleicher and Schuell Co (U S A) and Pharmacia Fine Chemicals (Sweden) respectively DEAE-cellulose was washed sequentially with 1 M NaOH, water, 1 M HCl, water, 1 M NaOH and finally with water before equilibration with the buffer DEAE-Sephadex was allowed to swell in excess of water, washed sequentially with 0 5 M HCl, water, 0 5 M NaOH and finally with water before equilibration with the buffer

Preparation of APS APS was prepared according to the method of BADDILEY *et al* [13] from pyridine sulfur trioxide and AMP APS was isolated by descending chromatography on Whatman No 3 paper using the solvent system ethanol–1 M ammonium acetate (7 5 3, v/v) It was eluted from the paper with 0 02 M Tris–HCl buffer (pH 8 5) at 4° and concentrated under vacuum at 30°

Preparation of [^{35}S]PAPS Carrier-free [^{35}S]PAPS was synthesized using an enzyme preparation from rat liver The enzyme was prepared as described by BRUNNGRABER[14] with some modifications 8 g of the liver were homogenized with 24 ml of 1 15% KCl solution and centrifuged at 20 000 × *g* for 1 h at 4° The supernatant was subjected to $(NH_4)_2SO_4$ fractionation, and the precipitate obtained between 1 5 and 2 1 M concentration of $(NH_4)_2SO_4$ was dissolved in the minimum amount of 0 02 M Tris–HCl buffer (pH 7 4) and was desalted by passing through a Sephadex G-75 column (2 2 cm × 22 cm) previously equilibrated with the same buffer The elution was carried out with the same buffer and the fractions at the void volume were collected, pooled and used for [^{35}S]PAPS synthesis The incubation mixture for the synthesis of [^{35}S]-PAPS consisted of 20 μmoles of Tris–HCl buffer (pH 8 0), 1 μmole of $MgCl_2$, 30 μC of carrier free $^{35}SO_4^{2-}$, 1 5 μmoles of ATP, 1 μmole of cysteine and 0 04 ml of the enzyme fraction in a total volume of 0 15 ml [^{35}S]PAPS was isolated from the reaction mixture by chromatography as described earlier[3] The [^{35}S]PAPS thus prepared contained about 0 3% of [^{35}S]APS and 15% $^{35}SO_4^{2-}$

Assay of the PAPS phosphohydrolase The assay mixture contained 30 μmoles of sodium acetate buffer (pH 5 0), [^{35}S]PAPS (about 600 000 counts/min corresponding to 1 9 pmoles) and the enzyme in a total volume of 0 15 ml After incubation for 1 h at 37°, the reaction was stopped by heating in a boiling-water bath for 1 min Immediately after heating, the mixture was cooled in ice and the precipitated protein was removed by centrifugation An aliquot of the supernatant was streaked in the form of

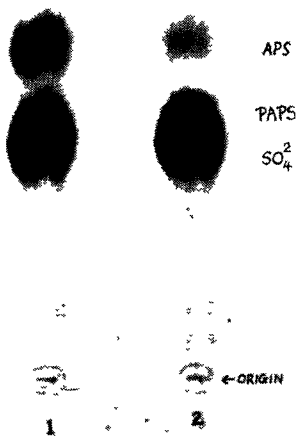

Fig 1 Radioautogram of the chromatographic separation of [^{35}S]APS from [^{35}S]PAPS and $^{35}SO_4^{2-}$ Conditions of assay were the same as described in the text 1 Reaction mixture containing 30 μmoles of sodium acetate buffer (pH 5 0), 600 000 counts/min of [^{35}S]PAPS and 0 03 mg of purified enzyme protein 2 Control reaction mixture

a 1-cm band on Whatman No 3 paper Descending chromatography was carried out using the solvent system[7] isobutyric acid–0 5 N ammonia (5 3, v/v) (Fig 1) The area corresponding to [^{35}S]APS was cut out from the paper and the radioactivity was measured in a Nuclear Chicago scintillation counter A control reaction mixture in which the enzyme was added at the end of the incubation period was run simultaneously Enough [^{35}S]PAPS was used in all reaction mixtures, and unreacted [^{35}S]PAPS could be detected at the end of the reaction in all the enzyme assays The radioactivity of [^{35}S]APS was corrected for absorption by the paper

Assay for 3'-nucleotidase The reaction mixture consisted of 50 μmoles of sodium acetate buffer (pH 5 0), 0 5 μmole of 3'-AMP, 0 8 μmole of $CoCl_2$ and the enzyme in a total volume of 0 3 ml After incubation for 30 min at 37°, 1 ml of 10% trichloroacetic acid was added to the mixture, the precipitated protein was removed by centrifugation and P_i released was estimated by the method of FISKE AND SUBBAROW[15]

PAPS sulfohydrolase was assayed as described by BALASUBRAMANIAN AND BACHHAWAT[5]

Protein was estimated according to LOWRY *et al* [16]

Preparation of PAPS phosphohydrolase All operations were carried out at 0–4

Extraction Fresh sheep brain cut into small pieces (100 g) was homogenized with 200 ml of 0 1 M Tris–HCl buffer (pH 7 4) in an ultra-Turrax homogenizer The

homogenate was centrifuged at 12 000 × *g* for 20 min, and the supernatant was dialyzed against 1 l of 0 1 M Tris–HCl (pH 7 4) for 4 h

$(NH_4)_2SO_4$ fractionation The dialyzed supernatant was brought to an $(NH_4)_2SO_4$ saturation of 40% by the addition of solid $(NH_4)_2SO_4$ with stirring and centrifuged at 12 000 × *g* for 20 min The precipitate was discarded The supernatant was further treated with $(NH_4)_2SO_4$ to a saturation of 60% and centrifuged at 12 000 × *g* for 20 min The precipitate was dissolved in 12 ml of 0 01 M Tris–HCl buffer (pH 7 4) and dialyzed against 500 ml of the same buffer for 4 h

DEAE-cellulose chromatography The dialyzed preparation was applied on a DEAE-cellulose column (15 cm × 1 5 cm) equilibrated with 0 01 M Tris–HCl (pH 7 4) The column was washed with 200 ml of the same buffer, and fractions of 15 ml were collected The first five fractions containing enzyme activity were pooled and $(NH_4)_2SO_4$ was added to 90% saturation The precipitate was collected by centrifugation and dissolved in 1 ml of 0 01 M Tris–HCl (pH 7 4) and dialyzed against 500 ml of the same buffer overnight

DEAE-Sephadex chromatography The DEAE-cellulose fraction was applied on a DEAE-Sephadex column (6 5 cm × 0 7 cm) equilibrated with 0 01 M Tris–HCl buffer (pH 7 4) The column was washed with 20 ml of the same buffer and to the washings $(NH_4)_2SO_4$ was added to 90% saturation The precipitate was collected by centrifugation, dissolved in 1 ml of 0 01 M Tris–HCl (pH 7 4) and dialyzed against 500 ml of the same buffer overnight

RESULTS

Purification of the enzyme

Table I shows a summary of the purification of the enzyme The procedure resulted in a 13-fold increase in specific activity of the enzyme with a recovery of 4% of the original activity Modifications of the procedure or other processes of purification, involving calcium phosphate gel treatment, heat treatment or acid precipitation, were tried without success as they did not improve upon the recovery or the increase in specific activity Elution with various concentrations of NaCl resulted in the recovery of smaller quantities of the enzyme from the DEAE-cellulose and DEAE-

TABLE I

PURIFICATION OF PAPS PHOSPHOHYDROLASE

Conditions of assay were the same as described in the text

Fraction	*Volume (ml)*	*Total units* 10^3*	*Total protein (mg)*	*Specific activity (units/mg protein) × 10^6*	*Yield (%)*
Crude (12 000 × *g* supernatant)	165	1 15	1423	0 81	100
$(NH_4)_2SO_4$ fraction	13	0 275	172	1 6	24
DEAE-cellulose fraction	2 5	0 067	10	6 7	6
DEAE-Sephadex fraction	2 5	0 049	4 5	10 9	4

* One enzyme unit is defined as 1 μmole of [^{35}S]APS formed in 1 h

Sephadex columns, however, the specific activity was decreased in the enzyme fractions. The major loss in the recovery of the enzyme occurred in the $(NH_4)_2SO_4$ fractionation and DEAE-cellulose chromatography steps The discarded fractions in these steps exhibited some PAPS phosphohydrolase activity of much lower specific activity The cause for the enzyme loss is not clearly understood, but it could result from the enrichment of certain interfering factors such as PAPS sulfohydrolase in these steps during the course of purification

The purified enzyme exhibited about 78% of its original activity after storage at $-18°$ for 2 weeks

Identification of the product of the reaction

The product of the reaction was identified as [^{35}S]APS by paper chromatography and electrophoresis The radioactive reaction product in the chromatographic assay was eluted from the paper by water at 4° and concentrated under vacuum The reaction product and the authentic sample of APS had the same R_F value (0 36) in the solvent system isobutyric acid–0 5 N ammonia (5 3, v/v) Cochromatography of the radioactive reaction product and authentic sample of APS on Whatman No 3 paper in the solvent system ethanol–1 M ammonium acetate (7 5 3, v/v) showed that the radioactivity coincided with the ultraviolet-quenching area of authentic APS (Fig 2)

Electrophoresis on a 7 5 cm × 37 5 cm strip of Whatman No 3 paper was carried out using a mixture of equal volumes[7] of 0 1 M Tris–acetate buffer (pH 6 5) and 0 05 M

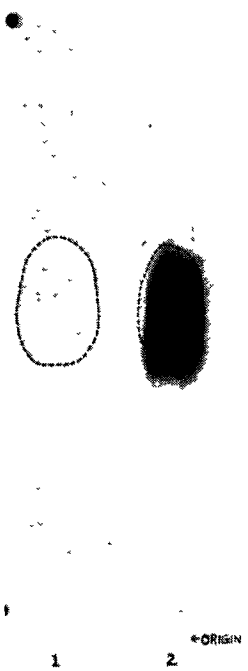

Fig 2 Paper chromatography of the radioactive product and authentic sample of APS Dotted lines outline the ultraviolet-quenching area of authentic APS, and the dark spot indicates the radioactive product 1 Authentic APS 2 A mixture of authentic APS and radioactive product

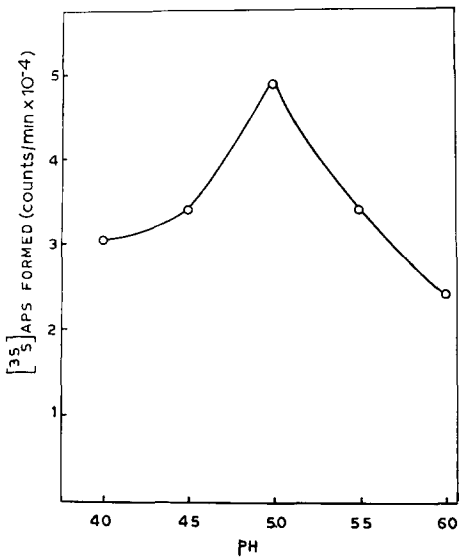

Fig 3 pH optimum of PAPS phosphohydrolase Assay conditions were the same as described in the text except that sodium acetate buffer of various pH's were used as shown in the figure. [^{35}S]PAPS 600 000 counts/min and 0 03 mg enzyme were used

citrate–phosphate buffer (pH 6 5) for 2 h at 600 V Under these conditions the distances moved by the radioactive product, [^{35}S]PAPS and $^{35}SO_4^{2-}$ were 10, 14 and 23 5 cm, respectively The mobility of the radioactive product was the same as that of authentic sample of APS

pH optimum and time-course of the reaction

The optimal pH for the enzymatic dephosphorylation of PAPS was pH 5 0 (Fig 3) The [^{35}S]APS formation showed a linear increase with time up to a period of 60 min, under the usual conditions of assay (Fig 4)

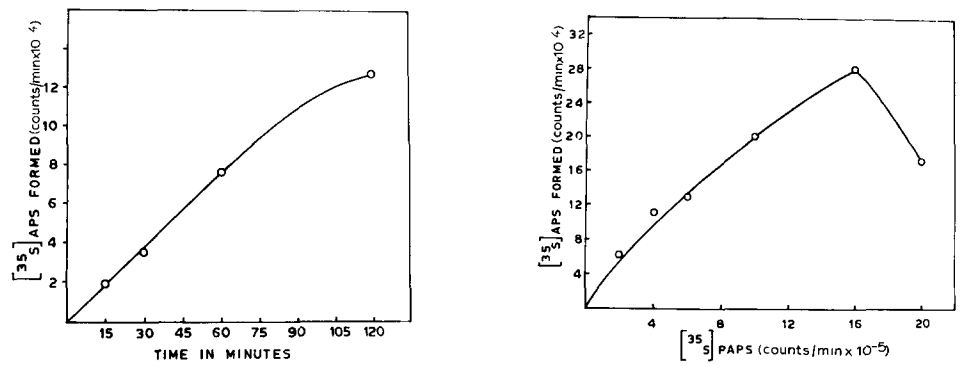

Fig 4 The time-course of [^{35}S]APS formation Assay conditions were the same as described in the text except that incubation period was varied as shown in the figure [^{35}S]PAPS 600 000 counts/min and 0 007 mg of enzyme protein were used

Fig 5 Effect of substrate concentration on [^{35}S]APS formation Assay conditions were the same as described in the text except that [^{35}S]PAPS concentration was varied as indicated in the figure 0 007 mg of enzyme was used

TABLE II

EFFECT OF METAL IONS AND EDTA ON PAPS PHOSPHOHYDROLASE

Assay mixture consisted of 2 μmoles of each addition, 30 μmoles of acetate buffer (pH 5 5), 600 000 counts/min of [^{35}S]PAPS and 0 09 mg of enzyme protein in a total volume of 0 15 ml Assay conditions were the same as described in the text

Addition	[^{35}S]*APS formed (counts/min)*
None	58 660
EDTA	98 460
$MgCl_2$	46 710
$CoCl_2$	12 510
$MnCl_2$	7 330

Effect of substrate concentration

The variation of [^{35}S]APS formation with increasing amounts of [^{35}S]PAPS is shown in Fig 5 There was an increase up to $1\,6 \times 10^6$ counts/min of [^{35}S]- PAPS used However, with $2\,0\ 10^6$ counts/min of [^{35}S]PAPS, there was a sharp decrease in enzyme activity, and with further increase in [^{35}S]PAPS, the activity declined.

EDTA and metal ion requirements of PAPS phosphohydrolase

Metal ions such as Mg^{2+}, Co^{2+} and Mn^{2+} were inhibitory, but EDTA stimulated the reaction (Table II) In contrast, the PAPS sulfohydrolase of brain has been reported to be activated by Co^{2+} and Mn^{2+} (ref 5) 3′-Nucleotidase activity of brain was found to be stimulated by Co^{2-}, Mg^{2+} and Mn^{2+}, as described below

Effect of nucleotides and other substances on PAPS phosphohydrolase

The effect of some nucleotides, –SH compounds and NaF on the enzyme activity is shown in Table III ADP and 3′-AMP were without significant influence on the enzyme activity ADP is known to be a powerful inhibitor of brain PAPS sulfohydrolase[5]

TABLE III

EFFECT OF NUCLEOTIDES AND OTHER SUBSTANCES ON PAPS PHOSPHOHYDROLASE

Assay mixture consisted of 30 μmoles of acetate buffer (pH 5 5), 2 μmoles of each addition, 600 000 counts/min of [^{35}S]PAPS and 0 09 mg of enzyme protein Assay conditions are described in the text

Addition	[^{35}S]*APS formed (counts/min)*
None	53 320
ADP	51 300
3′-AMP	51 310
3′-Phosphoadenosine 5′-phosphate	24 300
GSH	48 140
2,3-Dimercaptopropanol	43 100
NaF	0

3′-Phosphoadenosine 5′-phosphate was a powerful inhibitor of the enzyme The inhibition may be due to the close structural similarity of 3′-phosphoadenosine 5′-phosphate to PAPS GSH and 2,3-dimercaptopropanol depressed the enzyme activity NaF completely inhibited the reaction

3′-Nucleotidase and PAPS sulfohydrolase of brain

3′-Nucleotidase and PAPS sulfohydrolase activities were present in the purified enzyme preparation The recovery of these two enzyme activities from the crude extract was 0 4 and 0 02%, respectively, as compared to the value of 4% for the PAPS phosphohydrolase Since it has been reported[10] that 3′-nucleotidase can convert PAPS to APS, the properties of this enzyme activity in the purified fraction were studied in detail, using 3′-AMP as substrate The nucleotidase showed an optimum pH of 5 0 Metal ions like Co^{2+}, Ni^{2+}, Mn^{2+} and Mg^{2+} markedly stimulated the enzyme activity, and EDTA was slightly inhibitory (Table IV) This observation points to the possibility that the 3′-nucleotidase may be different from the PAPS phosphohydrolase In support of this idea was the observation that 3′-AMP did not inhibit the PAPS phosphohydrolase activity (Table III)

The PAPS phosphohydrolase of sheep brain exhibits widely different properties when compared to the PAPS sulfohydrolase also The latter enzyme has an optimum pH of 6 0, it is markedly activated by Co^{2+} and strongly inhibited by ADP (ref 5)

During the purification of the enzyme from the crude extract, the specific activity of PAPS phosphohydrolase increased to about 13-fold in the purified DEAE-Sephadex fraction However, the specific activity of PAPS sulfohydrolase decreased and that of 3′-nucleotidase increased to only about 1 5 times in the purified preparation

TABLE IV

EFFECT OF METAL IONS AND EDTA ON 3′-NUCLEOTIDASE

The reaction mixture consisted of 50 μmoles of sodium acetate buffer (pH 5), 0 5 μmole of 3′-AMP, 0 8 μmole of each addition and 0 22 mg of enzyme protein in a total volume of 0 3 ml Assay was done as described in the text

Addition	*Phosphate liberated (μmoles)*
None	0 019
$CoCl_2$	0 065
$NiCl_2$	0 035
$MgCl_2$	0 035
$MnCl_2$	0 029
EDTA	0 016

The ratio of specific activities of 3′-nucleotidase and PAPS sulfohydrolase to the specific activity of the phosphohydrolase in the crude and purified enzyme fractions is shown in Table V It is seen that the values are much lower for the purified enzyme fraction, compared to the values for the crude extract These considerations evidently point out that the PAPS phosphohydrolase exhibits characteristics much different from those of the 3′-nucleotidase and PAPS sulfohydrolase and indicate the possibility that different enzymes are responsible for these three activities in the brain

TABLE V

THE RATIO OF SPECIFIC ACTIVITIES OF PAPS PHOSPHOHYDROLASE, PAPS SULFOHYDROLASE AND 3′-NUCLEOTIDASE IN THE CRUDE AND PURIFIED ENZYME FRACTIONS

The purification procedure of the enzyme was as described in the text

Enzyme fraction	*Ratio of specific activities*	
	*3′-Nucleotidase** / *PAPS phosphohydrolase***	*PAPS sulfohydrolase**** / *PAPS phosphohydrolase*
Crude (12 000 × *g* supernatant)	0.617	1.09
DEAE-Sephadex fraction	0.073	0.01

* Assay was done as described in the text. Unit of activity was expressed as one μmole of phosphate liberated from 3′-AMP in 30 min.

** Assay was as described in the text. Unit of activity and specific activity were as given in Table I. There was about 13-fold increase in specific activity in the DEAE-Sephadex fraction compared to the crude extract.

*** Assay was as described in the text. Unit of activity was defined as 1 μmole of $^{35}SO_4^{2-}$ liberated from [^{35}S]PAPS in 1 h.

DISCUSSION

The present study demonstrates the conversion of PAPS to APS catalyzed by a partially purified enzyme preparation from sheep brain. Although the purified PAPS phosphohydrolase preparation has some 3′-nucleotidase and PAPS sulfohydrolase activity, a study of the pH optimum, the influence of metal ions and the effect of nucleotides and other substances show that these three enzyme activities differ from each other in their characteristics and indicate the possibility that different enzymes are responsible for these three activities. Furthermore, the specific activity of the PAPS phosphohydrolase had increased about 13-fold from the crude to the purified enzyme preparation, but there was practically no increase in the specific activities of the PAPS sulfohydrolase and 3′-nucleotidase.

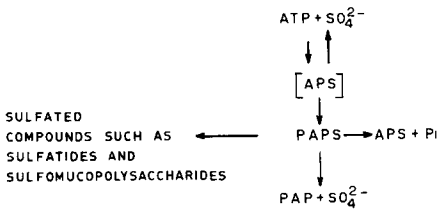

Fig. 6. Reactions leading to the synthesis and degradation of PAPS and sulfate transfer from PAPS in brain. PAP = 3′-phosphoadenosine 5′-phosphate.

From the present studies and the earlier work done in this laboratory[2–5], it is apparent that the concentration of PAPS is regulated by several enzymes in the brain. The various reactions catalyzed by these enzymes is shown in Fig. 6. While the importance of PAPS synthesis and sulfate transfer from PAPS to various acceptors is understandable, the significance of the PAPS-degrading enzymes such as PAPS

sulfohydrolase and PAPS phosphohydrolase is not quite clear The physiological role of these degrading enzymes may lie in the regulation of the concentration of PAPS or some of its degraded products such as APS, inorganic sulfate or 3'-phosphoadenosine 5'-phosphate The presence of a rat-liver sulfohydrolase enzyme acting on adenylylsulfate was recently reported by BAILEY-WOOD *et al* [17] We have also observed in preliminary experiments a weak sulfohydrolase activity in the purified brain enzyme preparation with [^{35}S]APS as substrate (pH 6 0)

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