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PURIFICATION AND PROPERTIES OF TWO ADENOSINE-5′-PHOSPHOSULPHATE SULPHOHYDROLASES FROM RAT LIVER AND THEIR POSSIBLE ROLE IN THE DEGRADATION OF 3′-PHOSPHOADENOSINE 5′-PHOSPHOSULPHATE

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SUMMARY

Enzymes capable of achieving the desulphation of 3′-phosphoadenosine 5′-phosphosulphate (PAPS) are widely distributed throughout the animal kingdom as well as within individual tissues of the rat. In high-speed supernatant preparations of the livers of M.R.C. hooded rats at least two routes are available for the desulphation of PAPS. The direct route involves a PAPS sulphohydrolase, the other depends on the initial formation of adenosine 5′-phosphosulphate (APS) by a Co^{2+}-activated 3′-nucleotidase followed by desulphation of the product by a specific APS sulphohydrolase. The latter enzyme has been considerably purified and its properties compared with those of a similar enzyme that has been partially purified from rat liver lysosomes. There is good evidence to suggest that the two enzymes are not identical. The lysosomal enzyme preparation also retains the ability to produce APS from PAPS. However, Co^{2+} has no effect on this process and the responsible enzyme may be a PAPS phosphohydrolase similar to the ones recently described by other workers.

INTRODUCTION

Active sulphate (3′-phosphoadenylyl sulphate, 3-phosphoadenosine 5′-phosphosulphate, PAPS) is generally accepted to be the sulphate donor in the biological sulphation of a wide range of compounds such as steroids, phenols and acid glycosaminoglycans. The immediate biosynthetic precursor of PAPS is adenylyl sulphate (adenosine 5′-phosphosulphate, APS) and, in mammalian livers, the enzymes responsible for the biosynthesis of the two compounds are localized in the soluble fraction of the liver cell[1,2].

Mammalian tissues also contain enzyme systems which are capable of degrading PAPS and APS, including a 3′-nucleotidase which dephosphorylates PAPS to

Abbreviations: PAPS, 3′-phosphoadenosine 5′-phosphosulphate; APS, adenosine 5′-phosphosulphate.

produce APS[3], a cobalt-activated[4] PAPS sulphohydrolase liberating inorganic sulphate from PAPS[5–7], an APS sulphohydrolase liberating inorganic sulphate from APS[8,9] as well as a number of deaminases which participate in the degradation of the purine ring[10].

It is not yet possible to present a complete picture of the intracellular distribution of these various enzymes and some of the results reported have been of a conflicting nature. Thus BRUNNGRABER[3] and SPENCER[8] have reported that PAPS is degraded by rat liver high-speed supernatant preparations. In contrast, KOIZUMI *et al.*[7], employing a different strain of rats, claim that no such degradation occurs unless the animals are first subjected to chronic liver damage or to hypervitaminosis A, leading to the release of a lysosomal PAPS sulphohydrolase. However, it seems likely that PAPS degradation by one route or another may be a feature of most of the sub-cellular fractions of the liver cell as well as cells from other tissues[6].

The present work confirms the observations of SPENCER[8] that PAPS can be degraded by the soluble fraction of the livers of M.R.C. hooded rats and describes the purification and properties of a highly specific APS sulphohydrolase from the soluble fraction of the liver cell which, together with a Co^{2+}-activated 3'-nucleotidase and a PAPS sulphohydrolase, participates in the degradative process. APS sulphohydrolase activity has also been found in rat liver lysosomes but evidence is presented that suggests that it is not the same enzyme as that present in the soluble fraction of the cell. A preliminary account of the soluble APS sulphohydrolase has previously been given[9].

MATERIALS AND METHODS

Experimental animals

M.R.C. hooded rats, aged 3–6 months, were used throughout. Other species studied were purchased or collected locally.

Preparation of [^{35}S]PAPS

The method used was an enzymic one, based on experimental conditions established in these laboratories by MATTOCK[11], material being purified by a modification of the procedure of BANERJEE AND ROY[12]. A 5.0-ml portion of a high-speed supernatant preparation of rat liver (20% homogenate in 0.25 M sucrose) was incubated for 1.5 h at 37° with 0.5 ml of 0.02 M K_2SO_4, 0.5 ml of 0.2 M $MgCl_2$, 2.0 ml of 0.2 M ATP and 100 μl of carrier-free $Na_2{}^{35}SO_4$, the final pH having been adjusted to 7.5. The enzyme reaction was terminated by heating to 100° for 30 sec, precipitated protein was removed by centrifuging at 2° and washed once by re-suspending in 10 ml of ice-cold water and re-centrifuging. The supernatant and washings were applied to a 15 cm × 3.5 cm column of Dowex-1 X8 (200–400 mesh, Cl^- form) which had been washed thoroughly with 4 M HCl followed by water. The column was eluted with 200 ml of water followed by 0.55 M NaCl in order to remove excess inorganic sulphate, ATP and traces of other nucleotides. Elution was continued until the $A_{260\ m\mu}$ of the eluate fell below 0.01. [^{35}S]PAPS was then eluted with 1.0 M NaCl (usually about 1 l was required). Norit A charcoal (0.3 g, washed as described by CHERNIAK AND DAVIDSON[13]) was added to the eluate and, after standing for 30 min with occasional stirring, the charcoal was filtered off *in vacuo* through two thicknesses

of Whatman No. 42 filter paper. The charcoal was washed with 100 ml of ice-cold water and the adsorbed [^{35}S]PAPS was then eluted with five 50-ml portions of aqueous ethanol (50%, v/v) containing 1% (v/v) of 1.0 M NH_4OH. Colloidal charcoal was removed from the eluate by filtering through a Sartorious CF-dense membrane and the filtrate was concentrated *in vacuo* at 30° to approx. 10 ml, portions of which could subsequently be diluted to required concentrations. Portions were stored at −15° and there was a loss of about 10% of the sulphated nucleotide (as inorganic $^{35}SO_4^{2-}$) over a period of 6 weeks. Fresh material was homogeneous on paper electrophoresis and chromatography and the assay of its concentration by the method of SPENCER[8] and by absorbance at 260 mμ gave results which were in good agreement.

Preparation of APS

The method of CHERNIAK AND DAVIDSON[13] was followed except that a double quantity of mono(tri-octyl)ammonium AMP was employed and the sulphation reaction was allowed to proceed at 4° in order to minimize the formation of products other than APS. The final product was stored in aqueous solution at −15° but decomposed appreciably after a period of 2–3 weeks.

Assay of [^{35}S]PAPS degradation by high-speed supernatant preparations of rat liver and other tissue preparations

High-speed supernatant preparations of rat liver, kidney, spleen, brain and lung were routinely prepared as follows. Rats were killed by a blow on the head and the tissue dissected out and washed in ice-cold 0.25 M sucrose solution before weighing and homogenizing in cold 0.25 M sucrose. The gap between the glass homogenization tube and the Teflon pestle (Tri-R homogenizer, Tri-R Instrument Co. Ltd., Rockville Centre, N.Y., U.S.A.) was 0.025 cm and the pestle was rotated at 4000 rev./min and forced through the suspension 6 times. The concentration of the homogenate was adjusted to 10 or 20% by adding further sucrose solution. After centrifuging at 81 000 × *g* for 45 min the clear supernatant was collected. High-speed supernatant preparations of gut, heart and skeletal muscle were obtained in the same way except that tissues were homogenized with the Sorvall Omni-mixer (Ivan Sorvall Inc., Conn., U.S.A.).

The standard incubation mixture consisted of 0.15 mM [^{35}S]PAPS, 5.0 mM $CoCl_2$, 0.25 M sodium acetate–acetic acid buffer (pH 5.2) and 20 μl of enzyme preparation in a total volume of 85 μl. After incubation for 30 min at 37° the reaction was stopped by heating to 100° for 30 sec. Suitable control determinations were performed in which enzyme was added to the other components immediately before terminating the reaction. Samples (10 μl) of test and control determinations were subjected to horizontal paper electrophoresis on Whatman No. 1 paper in 0.05 M sodium citrate–citric acid buffer (pH 5.8) at a potential gradient of 7.5 V/cm for 3 h. Radioactive areas on paper strips were located with the Packard Radiochromatogram Scanner, Model 7200 (Packard Instrument Co. Ill., U.S.A.) and peak areas corresponding to [^{35}S]PAPS, [^{35}S]APS and $^{35}SO_4^{2-}$ were determined in a manner analogous to that described by JONES AND DODGSON[14].

Assay of [^{35}S]PAPS degradation by high-speed supernatant preparations of other species

High-speed supernatant preparations were obtained as described in the pre-

ceding section except that the homogenization medium was isotonic KCl (11.4 g/l). The standard incubation mixture consisted of 0.075 mM [^{35}S]PAPS, 5.0 mM $CoCl_2$, 0.1 M buffer and 50 μl of enzyme preparation in a total volume of 105 μl. Sodium acetate–acetic acid buffer was used at pH 5.0 and 6.0 and Tris–HCl at pH 7.0, 8.0 and 9.0. In most experiments [^{35}S]PAPS, [^{35}S]APS and $^{35}SO_4^{2-}$ were determined after incubation but in other experiments only liberated $^{35}SO_4^{2-}$ was determined.

Assay of APS sulphohydrolase activity

The turbidimetric procedure (method B) of DODGSON[15] was modified. The method depends on the precipitation of enzymically liberated inorganic sulphate as a stabilized cloud of $BaSO_4$ in the presence of gelatin and trichloroacetic acid. However, APS interfered with the method, either due to its tendency to precipitate as the barium salt or to its instability in the presence of trichloroacetic acid. It was thus necessary to remove APS from incubation mixtures with charcoal before applying the turbidimetric method. Furthermore, when the method was employed to determine enzyme activity in relatively crude tissue preparations, addition of trichloroacetic acid as described in the original method led to the precipitation of large amounts of protein which interfered with the procedure. The original method was therefore modified as follows. The buffered enzyme–substrate incubation mixture was in a volume of 160 μl and, after incubation, 10–15 mg of Norit A charcoal was added with shaking and the whole kept in ice for 5 min. The suspension was centrifuged at 3000 rev./min for 1 min and then gently shaken in order to dislodge any charcoal floating on the meniscus before re-centrifuging for a further 1 min. A portion (100 μl) of the supernatant was mixed with 25 μl of 30% (w/v) trichloroacetic acid, precipitated protein was removed by centrifuging and a 100-μl portion of the clear supernatant mixed with 1.4 ml of a 4% solution of trichloroacetic acid. $BaCl_2$–gelatin reagent (0.5 ml) was then added and the procedure was then as originally described. The method operates over the range 0–40 μg SO_4^{2-} in the final 2.0 ml of solution. The accuracy of the modified method has been extensively checked in recovery experiments in which known amounts of K_2SO_4 were added to various tissue preparations (see also THOMAS[16]). When relatively pure enzyme preparations were assayed there was no need to remove protein prior to adding the reagent.

Assay of other enzymes

Other enzymes measured during the course of the work included rhodanese (EC 2.8.1.1)[17], glucose-6-phosphatase (EC 3.1.3.9)[18] and acid phosphatase (EC 3.1.3.2)[19] which were used as marker enzymes. PAPS-synthesizing enzymes were collectively assayed by a method based on that described earlier for the preparation of [^{35}S]PAPS, except that the procedure was scaled down to a total volume of 160 μl. After incubation, the reaction was stopped by heating at 100° for 30 sec, protein was removed by centrifuging and samples (10 μl) of the supernatants were subjected to horizontal paper electrophoresis as described earlier. Suitable control determinations were made. Paper strips were scanned and the percentage incorporation of $^{35}SO_4^{2-}$ into [^{35}S]PAPS determined from the relative areas of the appropriate peaks.

Determination of protein and RNA

Protein concentration was determined by the method of LOWRY *et al.*[20]

employing a standard calibration curve prepared with bovine serum albumin. RNA was determined by the method of MUNRO AND FLECK[21].

Cell fractionation procedure for rat liver

The procedure was based on that of DE DUVE *et al.*[18] starting with 10 g of liver obtained from two rats which had been starved for 12 h. All pellets were re-suspended in 0.25 M sucrose with the aid of a glass Dounce homogenizer, the nuclear pellet being diluted 1 to 5 (N fraction), the mitochondrial pellet 1 to 3 (M fraction), the lysosomal pellet 1 to 3 (L fraction), the microsomal fraction 1 to 4 (P fraction) whilst the soluble fraction was not diluted further (S fraction). Each fraction was assayed for APS sulphohydrolase and for appropriate marker enzymes, protein and RNA.

RESULTS

Degradation of [^{35}S]PAPS by high-speed supernatant preparations of various species

Table I shows that supernatant preparations from a wide variety of animal

TABLE I

DEGRADATION OF [^{35}S]PAPS BY HIGH-SPEED SUPERNATANT PREPARATIONS OF THE TISSUES OF VARIOUS SPECIES

Methods for preparation and assay of supernatants are given in the text. In many cases experiments were run at several pH values ranging from 5.0 to 9.0. The pH values quoted and marked with an asterisk are those at which maximum activity was obtained. The remaining values are arbitrary. The supernatants were prepared from 20% (w/v) homogenates except for the sea anemone (50%), flatworm (10%) and earthworm (30%). Where no values are quoted for [^{35}S]PAPS and [^{35}S]APS, liberated inorganic $^{35}SO_4^{2-}$ only was determined.

Species	*Tissue*	*pH*	*Relative percentage of radioactive components present after incubation*		
			(^{35}S)PAPS	*(^{35}S)APS*	$^{35}SO_4^{2-}$
Flatworm (Planaria)	Whole body	7.0	—	—	31
Sea anemone (Actinaria sp.)	Whole body	5.0*	0	0	100
Earthworm *(Lumbricus terrestis)*	Muscle	6.0*	0	0	100
Limpet *(Patella vulgaris)*	Digestive gland	6.0*	—	—	75
Mussel *(Mytelus rubens)*	Digestive gland	5.5*	—	—	63
Periwinkle *(Littorina littorea)*	Digestive gland	5.5*	0	0	100
Cockroach *(Blabberus crucifer)*	Hepatic caecae	7.5	—	—	87
Goldfish *(Carassius auratus)*	Liver	5.0	6	15	79
Frog *(Rana temporaria)*	Liver	5.0*	5	21	74
Toad *(Xenopus laevis)*	Liver	5.0*	0	22	78
Lizard *(Lacerta viridis)*	Liver	5.0	0	40	60
Chick *(Gallus domesticus)*	Liver	5.0	0	24	76

TABLE II

DEGRADATION OF [^{35}S]PAPS BY HIGH-SPEED SUPERNATANT PREPARATIONS OF VARIOUS RAT TISSUES

High-speed supernatant preparations (20%, w/v) of all tissues (except serum) were prepared in 0.25 M sucrose as described in the text. Serum was used without further dilution.

Tissue	*Relative percentage of radioactive components present after incubation*		
	(^{35}S)PAPS	*(^{35}S)APS*	*$^{35}SO_4^{2-}$*
Brain	0	67	33
Gut	0	36	64
Heart	63	27	10
Kidney	0	39	61
Liver	0	35	65
Lung	14	56	30
Muscle	75	17	8
Serum	84	7	9
Spleen	0	11	89

species are able to degrade [^{35}S]PAPS. In those cases where appropriate tests were made [^{35}S]APS was detected in addition to inorganic $^{35}SO_4^{2-}$ indicating the presence of a 3′-nucleotidase enzyme as well as one or more sulphohydrolase enzymes. When 15 mM ATP was included in the incubation mixture no degradation of [^{35}S]PAPS was observed.

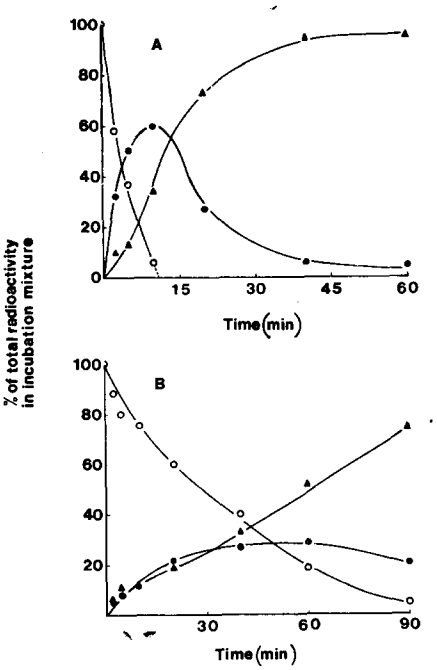


Fig. 1. Degradation of [^{35}S]PAPS by rat liver high-speed supernatant in the presence (A) and absence (B) of Co^{2+}. Incubations were performed in sodium acetate–acetic acid buffer (pH 5.2) and the per cent of the total radioactivity in the incubation mixture present as [^{35}S]PAPS (○—○), [^{35}S]APS (●—●) and $^{35}SO_4^{2-}$ (▲—▲) at various time intervals was determined as described in the text.

Degradation of [^{35}S]PAPS by high-speed supernatant preparations of various rat tissues

Table II shows that high-speed supernatant preparations of all the rat tissues examined were able to degrade [^{35}S]PAPS to produce [^{35}S]APS and inorganic $^{35}SO_4^{2-}$, and hence that all contained a 3'-nucleotidase enzyme as well as one or more sulphohydrolase.

Effect of cobalt on the degradation of [^{35}S]PAPS by rat liver high-speed supernatant preparations

Following the work of BALASUBRAMANIAN AND BACHHAWAT[4] on the PAPS-sulphohydrolase of sheep brain it seems to have been generally accepted that all such enzymes are activated by Co^{2+} and other workers in the field have always included the metal in their incubation mixtures. Fig. 1 shows the effect of the presence and absence of Co^{2+} on the time-course of the degradation of [^{35}S]PAPS by a rat liver high-speed supernatant preparation at pH 5.2. Co^{2+} accelerates the overall

TABLE III

APS SULPHOHYDROLASE ACTIVITY OF HIGH-SPEED SUPERNATANT PREPARATIONS OF VARIOUS RAT TISSUES

The preparation of the supernatants (20%, w/v) and other experimental conditions are described in the text.

Tissue	*Degree of hydrolysis of APS (%/h per mg protein)*
Brain	8.8
Gut	5.0
Heart	3.8
Kidney	4.4
Liver	8.0
Lung	6.4
Muscle	2.8
Serum	0.0
Spleen	5.9

process markedly but the curves clearly suggest that a major effect of the metal at this pH is in accelerating [^{35}S]APS production and hence in activating the 3'-nucleotidase enzyme. The subsequent rapid disappearance of [^{35}S]APS also indicates the presence of a sulphohydrolase which is active towards that nucleotide. The collective results further suggest that at pH 5.2 in the presence of Co^{2+} [^{35}S]PAPS degradation proceeds to a considerable extent through [^{35}S]APS, in which case the activity of a sulphohydrolase acting on the latter assumes considerable importance. For this reason it was decided to make a detailed study of this sulphohydrolase activity.

APS sulphohydrolase activity of high-speed supernatant preparations of various tissues

High-speed supernatant preparations from rat tissues also possessed sulphohydrolase activity towards APS (Table III). No activity could be detected in serum

under the experimental conditions in spite of the fact that some degradation of [^{35}S]PAPS could be achieved by this material (*cf.* Table II). Brain and liver supernatants appeared to be particularly good enzyme sources.

Intracellular distribution of APS sulphohydrolase activity in rat liver

Fig. 2 shows the intracellular distribution of APS sulphohydrolase activity in the liver cell in relation to that of marker enzymes and RNA. Rhodanese, acid

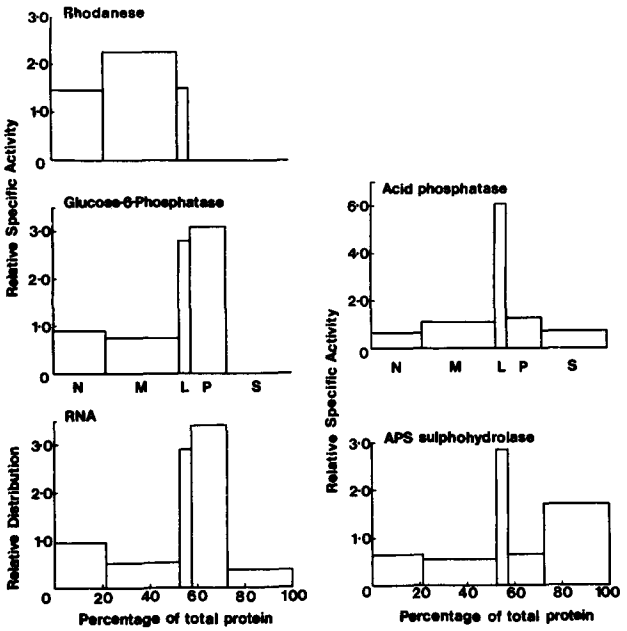


Fig. 2. The intracellular distribution of APS sulphohydrolase activity and various marker enzymes in the rat liver cell. Fractionation procedure and enzyme assays were as described in the text. N, M, L, P and S represent the nuclear, mitochondrial, lysosomal, microsomal and cell sap fractions, respectively.

phosphatase and glucose-6-phosphatase were employed as marker enzymes for the mitochondrial, lysosomal and microsomal fractions, respectively, and RNA was also used as a chemical marker for the latter fraction. The results were plotted as relative specific activity on the basis of protein according to the procedure of DE DUVE *et al.*[18]. Enzyme of high specific activity is present in the lysosomal fraction but considerable amounts of enzyme of lower specific activity are also present in the final supernatant fraction. Comparison of the distribution pattern with that of acid phosphatase suggests that the considerable quantities of enzyme activity present in the supernatant fraction are present *per se* and not as a result of lysosomal rupture and/or leakage during the fractionation procedure.

Attempts were subsequently made to purify APS sulphohydrolase activity from both supernatant and lysosomal fractions.

Purification of the high-speed supernatant enzyme

Stage 1. Livers from 20 rats (both sexes) were chilled and washed in ice-cold 0.25 M sucrose solution and were homogenized in a convenient volume of the sucrose solution by means of a Waring Blendor operated at maximum speed for 30 sec. The concentration of the suspension was adjusted to 20% (w/v) with further sucrose solution and centrifuged at 67 000 × *g* for 45 min at 2°. The clear supernatant was used immediately.

Stage 2. The pH of the supernatant was adjusted to 6.2 before heating (in batches of 200 ml contained in a large round-bottomed flask and gently swirled) in a water bath maintained at 55°. When the temperature of the solution approached 50° the flask was transferred for a period of 10 min (occasional stirring) to a second water bath maintained at 50°. After cooling to 10° the preparation was centrifuged at 20 000 × *g* for 10 min at 2° to remove precipitated protein. Most of the PAPS-synthesizing activity is eliminated at this stage.

Stage 3. Sufficient solid $(NH_4)_2SO_4$ was slowly added to the clear supernatant to give 40% saturation and after 3 h precipitated protein was separated and discarded. The concentration of $(NH_4)_2SO_4$ was then increased to 55% saturation, the precipitate separated, redissolved in the minimum volume of 0.01 M citrate buffer (pH 6.6) and dialysed overnight against 2 × 3 l of the same buffer.

Stage 4. The protein concentration of the Stage-3 preparation was determined and sufficient alumina Cγ gel was added to give a gel (dry weight)/protein ratio of 0.5. The suspension was allowed to stand at 2° for 30 min with occasional stirring and then centrifuged at 8000 × *g* for 10 min. The sedimented gel was dispersed in 12.5 ml of 0.25 M $(NH_4)_2SO_4$ solution and allowed to stand for 30 min before re-centrifuging. The clear supernatant was separated and retained whilst the sedimented gel was treated as before with a further 12.5 ml of $(NH_4)_2SO_4$ solution. After re-centrifuging, the two supernatants were pooled and sufficient solid $(NH_4)_2SO_4$ was added to give 80% saturation. After 3 h at 2° precipitated protein was separated by centrifuging at 26 000 × *g* for 30 min, redissolved in the minimum volume of 0.01 M citrate buffer (pH 6.6) and dialysed overnight against two changes of the same buffer as described above.

Stage 5. The Stage-4 preparation (usually about 60 mg of protein) was applied to a 1.5 cm × 10 cm column of DEAE-Sephadex which had been equilibrated with 0.01 M citrate buffer (pH 6.6). The column was then washed with the same buffer until the $A_{280\ m\mu}$ of the eluate fell below 0.1. Considerable amounts of unwanted protein were eliminated in this way. Elution was then continued with a linear gradient of the same buffer between the limits 0.01 and 0.1 M at a flow rate of 1.5 ml/min. Fractions (2 ml) were collected and assayed for protein and enzyme and appropriate ones were pooled and stored in small portions in the frozen state. The purification achieved with respect to the high-speed supernatant fraction was about 140-fold with a recovery of about 16% of the enzyme (Table IV). The various stages were tested for PAPS-synthesizing activity and for PAPS-degrading activity (at pH 5.2 and 6.0, in presence and absence of Co^{2+}). The former had virtually disappeared at Stage 2 and was not present at Stage 3 whilst PAPS-degrading activity, giving both APS and SO_4^{2-}, persisted through to Stage 4, but was absent from Stage 5. The final preparation was completely devoid of either 3'-nucleotidase or sulphohydrolase activity towards [^{35}S]PAPS. In addition, no ATPase activity could be detected.

TABLE IV

SUMMARY OF PURIFICATION SCHEMES FOR RAT LIVER APS SULPHOHYDROLASES FROM HIGH-SPEED SUPERNATANT (A) AND LYSOSOMES (B)

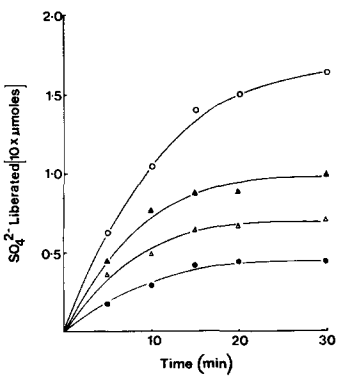
Assay conditions are as described in the text and one unit of enzyme activity is defined as that liberating 1 μmole of SO_4^{2-} from APS in 1 min.

Stage	*Procedure*	*Total activity (units)*	*Specific activity (units/mg protein)*	*Purification*	*Recovery (%)*
1	(A) High-speed supernatant	69	0.027	1.0	100
2	Heat treatment (50°)	55	0.029	1.1	80
3	$(NH_4)_2SO_4$ fraction (40–55%)	29	0.09	3.3	42
4	Alumina Cγ gel	23	0.4	14.8	33
5	DEAE-Sephadex	11	3.7	137	16
1	(B) Whole lysosomes	2.1	0.009	1.0	100
2	Lysosomal supernatant	1.9	0.034	3.8	94
3	Alumina Cγ gel	1.43	0.079	8.8	69
4	DEAE-Sephadex	0.75	0.50	56	36

Properties of the Stage-5 enzyme

pH stability. APS sulphohydrolase was relatively unstable at its optimum pH, even in the presence of substrate and this was reflected in typically anomalous time–activity and enzyme-concentration curves (see, for example, Fig. 3). Fig. 4 shows the effects of pre-incubating the enzyme at 37° for 10 min at various pH values before re-adjusting to the optimum pH and adding substrate. The enzyme is reasonably stable over the pH range 6.5–9.0 but becomes extremely unstable at lower pH and irreversibly inactivated at pH 4.0.

In all subsequent work with the Stage-5 preparation enzyme concentrations



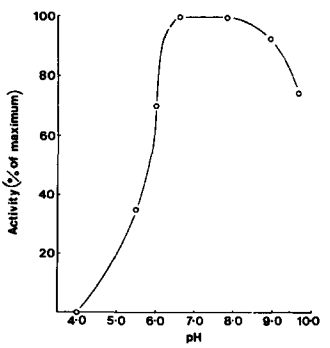


Fig. 3. Time–activity curves for the desulphation of APS at pH 5.2, by APS sulphohydrolase preparations of various protein concentrations: ○—○, 0.046 mg/ml; ▲—▲, 0.029 mg/ml; △—△, 0.023 mg/ml; ●—●, 0.019 mg/ml. Experimental details are described in the text.

Fig. 4. Effect of pH on the stability of the supernatant APS sulphohydrolase at 37°. The enzyme was incubated for 10 min at each pH followed by adjustment to pH 5.2 prior to assay of enzyme activity.

in incubation mixtures were so adjusted that incubation times of 5 min or less could be used.

Optimum pH and substrate concentration. The enzyme exhibited a sharp pH optimum of 5.2 in both 0.125 M sodium citrate–citric acid and 0.125 M sodium acetate–acetic acid buffers (Fig. 5). At the optimum pH in acetate buffer maximum activity was obtained at an APS concentration of approx. 7.5 mM (K_m = 1.0 mM).

Products of action of APS sulphohydrolase. Paper electrophoresis and paper chromatography (in three different solvent systems) of samples of enzyme–substrate mixtures after incubation showed that AMP was the sole nucleotide product of the enzymic action. The amount of AMP formed was estimated by eluting the appropriate area from electrophoresis strips and measurement of the $A_{260\ m\mu}$ against a suitable control. Determination of liberated SO_4^{2-} in a parallel sample of incubation mixture showed that the ratio of SO_4^{2-}/AMP was 0.91–1.0. The enzymic action thus involves the simple rupture of the phosphosulphate (P–O–S) link of APS.

Effects of various compounds on enzyme activity. Table V records the effects of metal ions, certain anions, nucleotides and certain so-called "group-specific reagents" on enzyme activity. The inhibitory effect of Co^{2+} is particularly interesting in the light of the claim[4] that PAPS sulphohydrolase is markedly activated by the metal. PP_i and P_i inhibit enzyme activity quite strongly and both are produced during the biosynthesis of PAPS by liver supernatant preparations. Various nucleotides inhibit

TABLE V

EFFECT OF VARIOUS COMPOUNDS ON THE ACTIVITY OF THE PURIFIED SUPERNATANT APS SULPHOHYDROLASE

Purified enzyme was preincubated with the compounds for 5 min before the addition of substrate. Incubation conditions were as described in the text.

Compound	*Concn. (mM)*	*Inhibition (%)*
Mg^{2+}	0.1	8
Ca^{2+}	0.1	15
Co^{2+}	0.1	20
Mn^{2+}	0.1	6
Cu^{2+}	0.1	76
Zn^{2+}	0.1	15
Fe^{2+}	0.1	30
Pb^{2+}	0.1	23
Hg^{2+}	0.1	100
P_i	10.0	55
SO_4^{2-}	10.0	7
Cl^-	10.0	4
PP_i	10.0	72
F^-	10.0	24
AMP	1.0	9
ADP	1.0	83
ATP	0.1	70
ITP	0.1	23
GTP	0.1	64
5,5′-Dithiobis(2-nitrobenzoate)	0.01	25
p-Hydroxymercuribenzoate	0.01	46
Hydroxylamine	0.1	40

the activity of the enzyme but the effect of ATP is particularly striking, 70% competitive inhibition occurring with 0.1 mM ATP. In contrast, the products of enzyme action (AMP and SO_4^{2-}) have little effect on enzyme activity.

Purification of the lysosomal enzyme

Stage 1. The livers from ten rats (both sexes and kept without food for 12 h) were washed in ice-cold 0.25 M sucrose–EDTA solution and homogenized with a convenient volume of the same sucrose–EDTA solution (Waring Blendor, operated at maximum speed for 30 sec). The concentration of the final homogenate was adjusted to 10% (w/v) by addition of further sucrose–EDTA solution. The method of RAGAB *et al.*[22] was then employed for the preparation of lysosomes (yield 200–300 mg of protein) which were then re-suspended in approx. 20 ml of 0.01 M citrate (pH 6.6).

Stage 2. The suspension was rapidly frozen and thawed 10 times and the whole was centrifuged at 59 000 × *g* for 30 min. The clear, yellow-brown supernatant was carefully separated and its protein concentration determined.

Stage 3. Sufficient alumina Cγ gel was added to the lysosomal extract to give a gel/protein ratio of 1.0 and the whole was allowed to stand for 10 min with occasional stirring. In contrast to the high-speed supernatant enzyme, the lysosomal enzyme was not adsorbed by the alumina gel, even at the higher gel concentration used. The gel was separated by centrifuging at 8000 × *g* for 10 min and discarded.

Stage 4. The enzyme was not adsorbed on DEAE-Sephadex under conditions in which the supernatant enzyme was adsorbed. The following procedure was devized: a column (12 cm × 0.9 cm) of DEAE-Sephadex (Cl^- form) was equilibrated with 0.01 M Tris–HCl buffer (pH 8.0). The enzyme preparation (20 ml containing about 25 mg of protein) was adjusted to pH 8.0 and applied to the column which was washed with further buffer until the $A_{280\ m\mu}$ fell below 0.1. The enzyme was eluted from the column with 30 ml of 0.01 M citrate buffer (pH 6.6) the solution was concentrated to approx. 5 ml by vacuum ultrafiltration and was then stored in small portions in the frozen state.

The purification generally achieved was about 60-fold from whole lysosomes with a 30% recovery (Table IV). Unfortunately, all stages retained the ability to degrade [^{35}S]PAPS and clearly 3′-nucleotidase is still present in the final preparation and possibly also a PAPS sulphohydrolase. It is of some interest that, under the conditions used, the presence or absence of Co^{2+} made little or no difference to the degradation of [^{35}S]PAPS by any of the stages.

Properties of the Stage-4 lysosomal enzyme

In contrast to the supernatant enzyme the lysosomal enzyme was stable at its optimum pH and an incubation period of 15 min was selected for subsequent studies.

Maximum enzyme activity was obtained at pH 5.0 in the presence of 0.125 M acetate buffer and 12.0 mM APS but the enzyme behaved quite differently from the supernatant enzyme in exhibiting high activity over a much wider range of pH (Fig. 5). The substrate concentration–activity curve was somewhat atypical and enzyme activity was still increasing at a concentration of 13.5 mM APS. However, the calculated K_m value was somewhat lower (0.87 mM) than that obtained for the supernatant enzyme. AMP and SO_4^{2-} were produced in equimolar amounts by the enzyme.

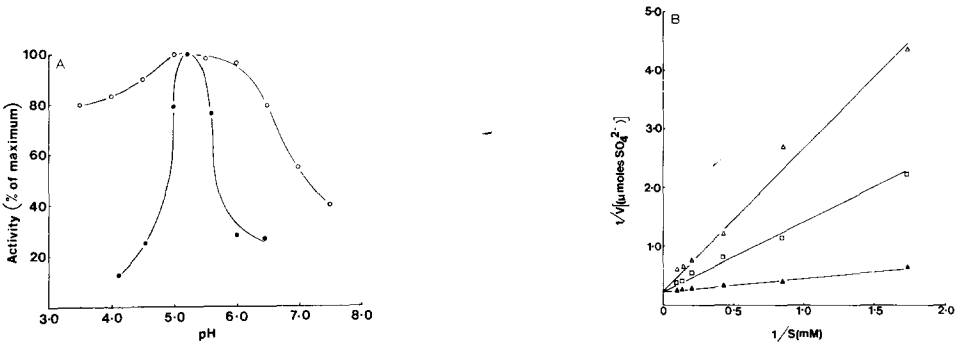


Fig. 5. A. Effect of pH on the activities of the supernatant (●—●) and lysosomal (○—○) APS sulphohydrolases in 0.125 M sodium acetate–acetic acid buffer. B. Lineweaver–Burk plots for the supernatant APS sulphohydrolase alone (▲—▲) and in the presence of 0.025 mM (□—□) and 0.05 mM (△—△) ATP.

Table VI summarizes the contrasting properties of the supernatant and lysosomal enzymes. Although firm proof must await further experimentation the indications are that lysosomal and supernatant activities towards APS reflect the activities of different enzymes.

Presence of PAPS sulphohydrolase in high-speed supernatant preparations

The collective results clearly establish the presence in rat liver high-speed supernatant preparations of a Co^{2+}-stimulated 3'-nucleotidase enzyme which is able to remove the 3'-phosphate group from PAPS to yield APS. Also present is a highly specific APS sulphohydrolase. Theoretically these two enzymes could collectively achieve the degradation of PAPS to AMP, inorganic sulphate and P_i. However, the question still remains as to whether a PAPS sulphohydrolase is also present in high-speed supernatant preparations. Fig. 6 shows the results of incubating such preparations for 30 min with [^{35}S]PAPS at a series of different pH values. Degradation of [^{35}]PAPS was greatest between pH 5.2 and 5.9 with maximum production of $^{35}SO_4^{2-}$ at the latter pH. The amount of [^{35}S]APS produced gradually decreased as the pH increased until at pH 8.0 very little was present at the end of the incubation.

TABLE VI

SUMMARY OF THE CONTRASTING PROPERTIES OF THE SUPERNATANT AND LYSOSOMAL APS SULPHOHYDROLASES

Treatment	*Supernatant enzyme*	*Lysosomal enzyme*
Alumina Cγ gel	Adsorbed	Not adsorbed
DEAE-Sephadex	Adsorbed	Not adsorbed
pH	Sharp pH optimum at 5.2	Broad pH optimum at 5.0
	Unstable at optimum pH	Stable at optimum pH
0.1 mM Co^{2+}	20% inhibition	8% inhibition
0.1 mM Cu^{2+}	76% inhibition	14% inhibition
10.0 mM P_i	55% inhibition	14% inhibition
10.0 mM PP_i	72% inhibition	11% inhibition
10.0 mM F^-	24% inhibition	0% inhibition

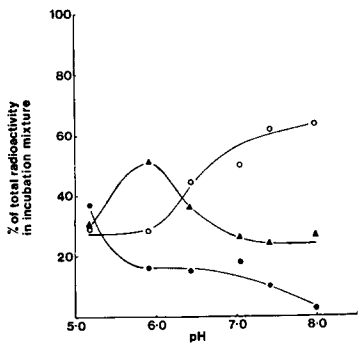


Fig. 6. Effect of pH on the degradation of [^{35}S]PAPS by high-speed supernatant of rat liver. Incubations were performed in sodium acetate–acetic acid buffer and the per cent of the total radioactivity in the incubation mixture present as [^{35}S]PAPS (○—○), [^{35}S]APS (●—●) and $^{35}SO_4^{2-}$ (▲—▲) after 10 min was determined as described in the text.

However, even at this pH appreciable amounts of $^{35}SO_4^{2-}$ were still produced. Since the activity of the supernatant APS sulphohydrolase must be extremely low at pH 8.0 (*cf.* Fig. 5) it seems probable that direct desulphation of [^{35}S]PAPS is occurring to a significant extent at this high pH.

Proof of this would normally involve the demonstration of PAP production but several difficulties are inherent in this. Thus direct transfer of sulphate from PAPS to endogenous acceptors present in liver supernatant preparations is well established[8] and leads to the appearance of PAP without the intervention of a sulphohydrolase. Furthermore, any PAP produced can also act as a substrate for the 3′-nucleotidase[3] so that a quantitative assessment of PAP formation becomes difficult. These and other problems were overcome in the present study and PAP production by desulphation of PAPS could be demonstrated by employing the Stage-3 preparation as enzyme source (endogenous acceptors are absent) and by incubating the preparation with PAPS in the presence of 0.04 M NaF (which inhibits PAP degradation by the 3′-nucleotidase). However the method finally developed was complex and it is difficult, spacewise, to justify a detailed account thereof. Moreover, it proved possible to resolve the problem in a much simpler way.

Briefly, APS sulphohydrolase activity in the Stage-3 supernatant preparation was completely eliminated by incubating the preparation at pH 4.0 and 37° for 10 min (see Fig. 4). Although the treated preparation was completely devoid of activity towards APS it retained the ability to liberate appreciable amounts of $^{35}SO_4^{2-}$ from [^{35}S]PAPS. Thus, 50 μl of the treated preparation liberated 27% of the available ^{35}S as $^{35}SO_4^{2-}$ when incubated for 15 min at 37° with 0.15 mM [^{35}S]PAPS in a total volume of 130 μl of 0.1 M acetate buffer (pH 5.2).

DISCUSSION

The present work establishes that enzymes capable of achieving the desulphation of PAPS are widely distributed throughout the animal kingdom as well as within the individual tissues of the rat. In the livers of M.R.C. hooded rats desulphation

of PAPS can take place *via* at least two different routes. One of these involves direct desulphation to yield PAP and inorganic sulphate. This type of breakdown has been observed in a number of different species[4,6,7,23] and involves one or more PAPS sulphohydrolase enzyme. Activity can be detected in more than one type of subcellular organelle and collective results suggest that Co^{2+} exerts a pronounced activatory effect.

The second route involves the initial formation of APS as a result of 3'-nucleotidase (or other phosphohydrolase) activity, followed by desulphation of the product by APS sulphohydrase(s). This route appears to be the principal one for PAPS degradation in rat liver supernatant preparations under the conditions employed in the present work. The 3'-nucleotidase present in such preparations is markedly activated by Co^{2+}. A similar route may also operate in rat liver lysosomes although, in this case, Co^{2+} seems not to affect the system. In this connexion, FAROOQUI AND BALASUBRAMANIAN[24] have reported the presence of a PAPS phosphohydrolase (partially inhibited by Co^{2+}), as well as a Co^{2+}-activated 3'-nucleotidase in Tris–HCl extracts of sheep brain. AUSTIN *et al.*[6] have studied the subcellular distribution of phosphohydrolase activity towards PAPS in pig kidney but always included Co^{2+} in their incubation mixtures, so that it is not possible to deduce whether more than one enzyme was being studied.

The present work also shows that at least two APS sulphohydrolases are present in rat liver. The cytosol enzyme is highly specific towards APS, relatively unstable and has a sharply defined pH optimum. In contrast, the partially purified lysosomal preparation still retains ability to degrade PAPS, so that the specificity of the APS-desulphating component of the preparation cannot yet be stated with certainty. This enzyme exhibits several properties which distinguish it from the cytosol enzyme and one particular feature—the extremely broad and asymmetrical pH activity curve—leads one to suspect that more than one APS sulphohydrolase might be present (*cf.* the work of ARMSTRONG *et al.*[25] with pig kidney).

Clearly, a variety of complex systems exist in mammalian cells for the degradation of PAPS and these systems may vary from species to species. Presumably they have important roles to play in the control of sulphation processes within the cell and, in turn, must themselves be controlled. It is not without interest that the cytosol APS sulphohydrolase co-exists with the enzymes which are responsible for the biosynthesis of PAPS from ATP and inorganic sulphate. The relationship between the sulphohydrolase and the biosynthetic enzymes is not yet clear but the fact that ATP and ADP (respectively, a starting component and an end-product of PAPS biosynthesis) are powerful inhibitors of APS sulphohydrolase suggests that, under normal circumstances of PAPS biosynthesis, sulphohydrolase activity may be suppressed thus allowing biosynthesis to proceed.

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REFERENCES

1 P. W. ROBBINS AND F. LIPMANN, *J. Biol. Chem.*, 233 (1958) 681.
2 A. S. LEVI AND G. WOLF, *Biochim. Biophys. Acta*, 178 (1969) 262.

3 E. G. BRUNNGRABER, *J. Biol. Chem.*, 233 (1958) 472.
4 A. S. BALASUBRAMANIAN AND B. K. BACHHAWAT, *Biochim. Biophys. Acta*, 59 (1962) 389.
5 J. B. ADAMS, *Biochim. Biophys. Acta*, 83 (1964) 127.
6 J. AUSTIN, D. ARMSTRONG, D. STUMPF, T. LUTTENBEGGER AND M. DRAGOO, *Biochim. Biophys. Acta*, 192 (1969) 29.
7 T. KOIZUMI, T. SUETMATSU, A. KAWASAKI, K. HIRAMATSU AND H. ABE, *Biochim. Biophys. Acta*, 184 (1969) 106.
8 B. SPENCER, *Biochem. J.*, 77 (1960) 294.
9 R. BAILEY-WOOD, K. S. DODGSON AND F. A. ROSE, *Biochem. J.*, 112 (1969) 257.
10 M. H. R. LEWIS AND B. SPENCER, *Biochem. J.*, 85 (1962) 18P.
11 P. MATTOCK, *Some Aspects of Sulphate Activation and Transfer in Biological Systems*, Ph. D. Thesis, University of Wales, 1967.
12 R. K. BANNERJEE AND A. B. ROY, *Mol. Pharmacol.*, 2 (1966) 56.
13 R. CHERNIAK AND E. A. DAVIDSON, *J. Biol. Chem.*, 239 (1964) 2986.
14 J. G. JONES AND K. S. DODGSON, *Biochem. J.*, 94 (1965) 331.
15 K. S. DODGSON, *Biochem. J.*, 78 (1961) 312.
16 J. H. THOMAS, *The Chemical and Enzymic Desulphation of* L-*Serine O-Sulphate and Related Compounds*, Ph. D. Thesis, University of Wales, 1966.
17 O. ROSENTHAL, C. S. ROGERS, H. M. VARS AND C. C. FERGUSSON, *J. Biol. Chem.*, 185 (1950) 669.
18 C. DE DUVE, E. C. PRESSMAN, R. GIANETTO, R. WATTIAUX AND F. APPELMANS, *Biochem. J.*, 60 (1955) 604.
19 R. GIANETTO AND C. DE DUVE, *Biochem. J.*, 59 (1955) 433.
20 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
21 H. N. MUNRO AND A. FLECK, *Methods of Biochemical Analysis*, 14 (1966) 113.
22 H. RAGAB, C. BECK, C. DILLARD AND A. L. TAPPEL, *Biochim. Biophys. Acta*, 148 (1967) 501.
23 S. SUZUKI AND J. L. STROMINGER, *J. Biol. Chem.*, 235 (1960) 257.
24 A. A. FAROOQUI AND A. S. BALASUBRAMANIAN, *Biochim. Biophys. Acta*, 198 (1970) 56.
25 D. ARMSTRONG, J. AUSTIN, T. LUTTENEGGER, B. BACHHAWAT AND D. STUMPF, *Biochim. Biophys. Acta*, 198 (1970) 523.