Biochimica et Biophysica Acta, 527 (1978) 70—85

BBA 68593

PURIFICATION AND PROPERTIES OF BOVINE LIVER LYSOSOMAL ADENOSINE 5′-PHOSPHOSULPHATE SULPHOHYDROLASE

A NON-SPECIFIC ENZYME WITH PYROPHOSPHATASE AND PHOSPHODIESTERASE ACTIVITIES

KARL M. ROGERS *, GRAHAM F. WHITE and KENNETH S. DODGSON **

Department of Biochemistry, University College, P.O. Box 78, Cardiff CF1 1XL (U.K.)

(Received April 6th, 1978)

Summary

Bovine liver lysosomal adenosine 5′-phosphosulphate sulphohydrolase (EC 3.6.2.1) was purified to apparent homogeneity. The molecular weight of the enzyme was 53 000 by sodium dodecyl sulphate polyacrylamide gel electrophoresis and 56 000 by BioGel P-150 gel filtration. The substrate specificity of the enzyme was studied. The several substrates towards which the enzyme preparation showed high activity were used to establish that a single enzyme was responsible for the different activities. This multiple specificity provides a possible explanation of the physiological role of lysosomal adenosine 5′-phosphosulphate sulphohydrolase.

Introduction

The main sulphate donor in mammalian systems is 3′-phosphoadenosine 5′-phosphosulphate, the immediate biosynthetic precursor of which is adenosine 5′-phosphosulphate. The enzymes responsible for the synthesis of the latter compound and its conversion to 3′-phosphoadenosine 5′-phosphosulphate are localized in the soluble fraction of the cell [1,2]. In addition, the liver cytosol contains a number of distinct enzymes capable of degrading 'active sulphate' [3]. 3′-Phosphoadenosine 5′-phosphosulphate may thus be desulphated either by the direct action of 3′-phosphoadenosine 5′-phosphosulphate sulphohydrolase (EC 3.6.2.2) or by the sequential action of a 3′-nucleotidase (EC 3.1.3.6) and adenosine 5′-phosphosulphate sulphohydrolase

* Present address: School of Biochemistry, University of Melbourne, Parkville 3052, Victoria, Australia.
** To whom reprint requests should be addressed.

(EC 3.6.2.1). A study of these enzymes is important not only because they may be implicated in the control of sulphation reactions, but also because they may interfere in experimental systems designed for the study of biological sulphation reactions.

Earlier work from these laboratories has shown that in both rat liver [4] and bovine liver [5] adenosine 5′-phosphosulphate sulphohydrolase activity enjoys a bimodal distribution, being present not only in the cytosol but also in the lysosomal fraction. The cytosol enzyme has been purified from bovine liver [5] and its properties reported [6]. In view of the fact that lysosomes contain enzymes generally employed in the degradation of macromolecules, the presence of an additional enzyme in the lysosomes for the purpose of degrading a relatively small molecule like adenosine 5′-phosphosulphate seems rather curious. The present work was therefore undertaken in order to isolate and purify the lysosomal adenosine 5′-phosphosulphate sulphohydrolase, to report on its properties, and to shed some light on the role of this enzyme within the lysosomes.

Materials and Methods

With the exception of the material detailed below, all reagents and materials were obtained from either BDH Chemicals, Poole, Dorset, U.K., or Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Carrier-free $Na_2{}^{35}SO_4$ and [U-^{14}C]ATP (562 Ci/mol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. 2,5-Diphenyloxazole and Triton-X100 for liquid scintillation counting were obtained from Fisons Scientific Apparatus, Loughborough, Leics., U.K. Whatman DE-81 ion-exchange paper, Nos. 1 and 3 MM chromatography papers, and microgranular DE52 and CM52 ion-exchange celluloses were obtained through H. Reeve Angel and Co. Ltd., London, U.K. 5′-AMP-Sepharose 4B and BioGel P-150 were obtained from Pharmacia, Uppsala, Sweden, and BioRad Laboratories, Bromley, Kent, U.K., respectively.

Preparation of adenosine 5′-phosphosulphate and adenosine 5′-phospho[^{35}S]-sulphate

Adenosine 5′-phosphosulphate was prepared by the method of Cherniak and Davidson [7] as modified by Stokes et al. [5].

Adenosine 5′-phospho[^{35}S]sulphate was obtained by enzymic synthesis. The following incubation mixture was used. 1 μmol ATP, 1 μmol $MgCl_2$, 1 nmol K_2SO_4, 0.2 mCi carrier-free $Na_2{}^{35}SO_4$, 1.25 units ATP:sulphate adenyltransferase (EC 2.7.7.4) and 12.5 units of inorganic pyrophosphate phosphohydrolase (EC 3.6.1.1) in a total volume of 1 ml. After incubation at 37°C for 90 min, the reaction was terminated by heating at 100°C for 30 s, and subsequent immersion in ice-water. 2 μmol carrier adenosine 5′-phosphosulphate was added and the adenosine 5′-phospho[^{35}S]sulphate purified according to the procedure of Cherniak and Davidson [7] on an appropriately reduced scale. The final preparation was adjusted to 0.1—0.2 mM and contained approx. 100 Ci/mol. This preparation was diluted as required with carrier adenosine 5′-phosphosulphate in order to obtain the desired specific activity in the final working solutions.

Alternatively, crude preparations of adenosine 5′-phospho[^{35}S]sulphate and crude carrier were mixed and purified together in a single procedure in order to obtain the final working solutions directly. Purities of adenosine 5′-phospho[^{35}S]sulphate and carrier adenosine 5′-phosphosulphate were determined by ion-exchange paper chromatography and paper electrophoresis (see below). Freshly prepared samples showed no contamination with other nucleotides or ^{35}S-labelled compounds. Up to 10% hydrolysis to AMP and SO_4^{2-} occurred during storage of the frozen solution for 5—6 weeks. Consequently, freshly purified material was used whenever possible, and always for kinetic measurements.

Preparation of 3′-phosphoadenosine 5′-phospho[^{35}S]sulphate

3′-Phosphoadenosine 5′-phospho[^{35}S]sulphate was prepared by an enzymic method based on experimental conditions established in these laboratories [8] and was purified according to the procedure described by Bannerjee and Roy [9] and Bailey-Wood et al. [4].

Assay of enzymes

General comments. All enzyme assays were performed in duplicate at 37°C using freshly prepared solutions of ATP, PP_i and bis(4-nitrophenyl)phosphate, or freshly thawed solutions of adenosine 5′-phosphosulphate and 3′-phosphoadenosine 5′-phosphosulphate. During enzyme purification, controls were performed using the appropriate enzyme samples previously heated at 100°C for 2 min.

In kinetic studies, 25-μl portions of purified enzyme containing 3—8 μg protein were diluted with 100 μl ice-cold glycerol solution (62.5% v/v) immediately prior to use. This diluted enzyme, hereafter referred to as '50% glycerol/enzyme', retained 100% activity for at least 30 h at 0°C. Reproducibility of assays with 50% glycerol/enzyme was greatly improved by using polyethylene or polypropylene incubation tubes instead of glass. In order to allow accurate determination of initial reaction velocities, the incubation volumes in the standard methods described below were increased 4- or 5-fold, and three or four samples were taken for assay at different times. Controls contained 50% glycerol in place of enzyme solution.

Adenosine 5′-phosphosulphate sulphohydrolase. The procedure adopted for the assay of adenosine 5′-phosphosulphate sulphohydrolase was the radioisotopic version [5] of the method described by Bailey-Wood et al. [4]. Essentially, the method involved determination of the rate of release of $^{35}SO_4^{2-}$ from buffered solutions of adenosine 5′-phospho[^{35}S]sulphate at 37°C.

For routine assays during enzyme purification, incubation mixtures containing 5 mM adenosine 5′-phospho[^{35}S]sulphate (specific activity 0.5—1.0 · 10^5 DPM/μmol), 0.1 M acetic acid/NaOH buffer (pH 5.2) and enzyme in a total volume of 0.2 ml. After incubation for 1—20 min and removal of residual adenosine 5′-phospho[^{35}S]sulphate with activated Norit A charcoal, 50- or 100-μl samples of clear supernatant were counted for radioactivity in 10 ml of scintillant containing 2.5 g 2,5-diphenyloxazole per l toluene/2-methoxyethanol (4 : 1, v/v). One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol SO_4^{2-} from adenosine 5′-phosphosulphate

per min under these conditions. For some kinetic experiments when the adenosine 5′-phosphosulphate concentration was reduced (e.g., during the determination of K_m), the lower amounts of sulphate released contained too little radioactivity to permit accurate measurement. In these cases, the sample volume was increased to 1.0 ml and, after charcoal treatment, 0.7 ml supernatant solution was counted in 10 ml of scintillant containing 4 g 2,5-diphenyloxazole per l toluene/Triton-X100 (2 : 1, v/v).

3′-Phosphoadenosine 5′-phosphosulphate sulphohydrolase. The assay method was the same as for adenosine 5′-phosphosulphate sulphohydrolase, except that adenosine 5′-phospho[^{35}S]sulphate was replaced by 20 μM 3′-phosphoadenosine 5′-phospho[^{35}S]sulphate.

ATPase. Hydrolysis of ATP was measured by the rate of release of P_i from ATP. For routine assays, incubation mixtures contained 1 mM ATP, 0.1 M acetic acid/NaOH buffer (pH 5.2) and enzyme in a total volume of 0.2 ml contained in 10 ml tapered test tubes. After incubation at 37°C for 1—10 min, the reaction was stopped by adding 1.3 ml 10 mM HCl and the liberated P_i was determined by the method of Parvin and Smith [10].

For samples in the early stages of purification, the method was modified as follows in order to remove the large amounts of protein present which otherwise interfere in the molybdovanadate assay of phosphate. Trichloroacetic acid (1.3 ml, 2%) was added to the incubation mixture in place of 10 mM HCl, and precipitated protein was removed by centrifugation. A 1-ml sample of supernatant was then assayed as before, but using appropriately reduced volumes of the other reagents.

For kinetic studies with purified enzyme at lower concentrations of ATP, the sensitivity of the method was increased by mixing 200-μl samples of incubate with 50 μl 1 M HCl, followed by 0.5 ml butan-1-ol and 0.25 ml molybdovanadate reagent. The absorbance of the butanol layer was measured in a Hilger Uvispek spectrophotometer equipped with microcells (1-cm pathlength, 0.2-ml capacity). Calibration curves were constructed by the same method, using standard solutions of P_i. ADP-phosphohydrolase was assayed in an identical manner.

Inorganic pyrophosphatase. The standard method for the assay of pyrophosphatase activity was as described above for ATPase except that 1 mM ATP was replaced by 2.5 mM sodium pyrophosphate. All rates of reaction refer to the rate of disappearance of PP_i, calculated as one half of the rate of formation of P_i.

FAD-pyrophosphatase. Hydrolysis of FAD to FMN and AMP was assayed at pH 5.2 by measuring the accompanying increase in fluorescence at 530 nm, according to Ragab et al. [21].

Phosphomonoesterases. Activity towards *O*-phospho-D-serine, *O*-phospho-L-serine, 5′-AMP, 2′(3′)-AMP and glucose 6-phosphate was monitored by measuring P_i release by the methods described above.

Phosphodiesterase. This assay was based on the release of 4-nitrophenol from bis(4-nitrophenyl)phosphate. Incubation mixtures contained 5 mM bis(4-nitrophenyl)phosphate, 0.1 M acetic acid/NaOH buffer (pH 5.2) and enzyme in a total volume of 0.2 ml. After incubation for 5—10 min at 37°C, 1 ml 0.1 M NaOH was added and the extinction of the 4-nitrophenol anion measured at

400 nm. Activities towards 4-nitrophenyl 5'-phosphothymidine (phosphodiesterase I) and 4-nitrophenyl 3'-phosphothymidine (phosphodiesterase II) were measured in the same way, using 50 μM concentrations of substrates.

DNAase and RNAase. These activities were assayed as described by de Duve et al. [11] except that the pH was 5.2 and the DNAase assay mixture contained 0.2 M KCl.

Assay of protein

Protein concentrations were determined by the method of Lowry et al. [12] using bovine serum albumin as standard. Protein contents of eluates from chromatography columns were continuously monitored using absorbance at 280 nm.

Ion-exchange and gel filtration column chromatography

DEAE-cellulose (Whatman DE-52), CM-cellulose (Whatman CM-52) and BioGel P-150 were precycled according to the recommended procedures and washed extensively with the appropriate starting buffer before use.

Ion-exchange paper chromatography

Ion-exchange paper chromatography of substrates and products of hydrolysis was performed on Whatman DE-81 ion-exchange paper according to Denner and Stokes [13]. After drying the paper strips, nucleotides and compounds of 4-nitrophenol were located by their quenching of paper fluorescence when viewed under ultraviolet irradiation. Radioactive areas were located using a Packard Model 7200 Radiochromatogram Scanner.

Paper electrophoresis

Samples were spotted on Whatman No. 1 paper (40 X 12 cm) and run in either 0.025 M citric acid/NaOH (pH 5.8) or 0.05 M potassium dihydrogen phosphate/KOH buffer (pH 7.3) with a potential gradient of 10 V/cm for 3 h. Alternatively, Whatman 3MM paper (75 X 15 cm) was used for high-voltage electrophoresis on a Locarte apparatus (4 kV for 45 min).

Polyacrylamide gel electrophoresis

Enzyme samples for polyacrylamide gel electrophoresis were dialysed against 0.01 M acetic acid/NaOH buffer (pH 5.2). SDS gels were prepared and used according to the method of Weber et al. [14]. Acidic gel electrophoresis was performed in System III of Gabriel [15] using gels containing 7.5% acrylamide. Sometimes, with small sample volumes, the stacking gel was omitted. Gels were pre-electrophoresed in gel buffer for 2 h at 3 mA per tube. Electrophoretic runs were performed at 4°C for 2 h at 2 mA per tube, using methyl green as tracker dye. Basic gels, containing 7.5% acrylamide, were prepared and run according to System I of Gabriel [15]. Pre-electrophoresis and running conditions were as used with acidic gels and Bromophenol Blue was used as marker.

Protein bands were fixed in 25% trichloroacetic acid at 37°C overnight, and stained by immersion for 90 min in 0.25% Coomassie Brilliant Blue R250 in 50% trichloroacetic acid at 37°C. Subsequently gels were destained using many changes of 7% acetic acid.

Adenosine 5'-phosphosulphate sulphohydrolase activity was detected by assaying gel sections as follows. Extruded gels were cut into 2-mm sections, placed in 100 μl ice-cold water in 0.5 X 5.0 cm polypropylene test tubes, and dispersed by maceration with a small glass rod. Reaction was started by adding 100 μl 0.2 M acetic-acid/NaOH buffer (pH 5.9), containing 20 μM adenosine 5'-phospho[^{35}S]sulphate (specific activity approx. $5 \cdot 10^4$ dpm/nmol). After incubation at 37°C for 10 min, the reaction was stopped and the assay completed in the usual way.

For ATPase and pyrophosphatase, gels were dispersed as described above. Reaction was started by adding 150 μl solutions of ATP or PP_i in 0.167 M acetic acid/NaOH buffer (pH 5.9) to give final concentrations of 50 μM or 25 μM, respectively. After incubation at 37°C for 10 min, reaction was stopped by the addition of 60 μl 1 M HCl. After brief centrifugation to remove gel particles, 200-μl samples of the supernatant solutions were assayed for liberated P_i by the semimicro method described earlier. Phosphodiesterase activity was located on acidic gels by incubation in a solution containing 5 mM bis(4-nitrophenyl)phosphate and 0.1 M acetic acid/NaOH buffer (pH 5.9) at room temperature for 20 min. Bands were revealed by placing the gel in a stream of NH_3 gas whereupon the yellow colour of the liberated 4-nitrophenyl anion became apparent. Basic gels were incubated in 5 mM bis(4-nitrophenyl)phosphate in 0.1 M Tris-maleate (pH 6.8). The yellow band of 4-nitrophenyl anion became apparent without further treatment.

Purification of adenosine 5'-phosphosulphate sulphohydrolase

All procedures were carried out at 4°C and all centrifugations were performed in a 6 X 250 ml rotor (No. 69179) in an MSE High Speed 18 centrifuge unless otherwise indicated. The enzyme could be stored frozen at any stage during the purification without loss of activity.

Stage 1. Fresh bovine liver (from oxen not receiving tenderizer injections) was obtained from a local slaughterhouse and kept on ice during transportation to the laboratory. Batches (1 kg) were worked up as follows, until a lysosomal-mitochondrial suspension from 9 kg liver had been obtained.

Liver was freed from connective tissue, cut into small strips, finely chopped with a sharp knife and homogenized in 0.15 M KCl using a Waring Commercial Blender operated at maximum speed for 25 s to give a 30% (w/v) homogenate. This was centrifuged at 1000 X g_{av} for 10 min (6 X 1000 ml rotor No. 59560 in a MSE mistral 6L centrifuge) to remove intact cells and nuclei. The supernatant was centrifuged at 12 700 X g_{av} for 20 min. The resulting pellet was washed by resuspension in 0.15 M KCl (one half of the volume of the Mistral 6L supernatant), and retrieved by repeating the centrifugation. The washed pellet was suspended in 0.01 M citric acid/NaOH buffer, pH 5.5 (500 ml for the amount of pellet derived from 1.0 kg of liver), to give the lysosomal-mitochondrial suspension.

Stage 2. The suspension was frozen and thawed ten times in batches in order to release lysosomal enzymes.

Stage 3. After the tenth thawing, the suspension was centrifuged at 36 500 X g_{av} for 60 min (Beckman L2 ultracentrifuge, No. 19 rotor). The pellet was discarded.

Stage 4. Sufficient solid $(NH_4)_2SO_4$ was slowly added with stirring to the supernatant from stage 3 to produce 45% saturation (calculated from the nomogram of Dixon [16] without correction for temperature). After stirring for 30 min, the concentration of $(NH_4)_2SO_4$ was increased to 52% saturation. After a further 30 min of stirring, the precipitate was removed by centrifuging (12 700 $\times g_{av}$, 22 min) and discarded. The $(NH_4)_2SO_4$ concentration of the supernatant was adjusted to 72% saturation and after a further 3 h stirring the precipitate was recovered by centrifuging and resuspending in 10 mM Tris-HCl buffer, pH 8.4 (16 ml for the pellet derived from each l of stage 3 supernatant). The pH of the suspension was increased to 8.4 using 1 M NH_4OH and the whole dialysed against several changes of 0.01 M Tris-HCl buffer, pH 8.4. A small precipitate was removed by centrifuging and discarded.

Stage 5. The stage 4 preparation was applied to a 9 cm (length) $\times$ 11 cm (diameter) column of microgranular DEAE-cellulose which had been equilibrated with 0.01 M Tris-HCl buffer (pH 8.4). The column was washed with the same buffer and the effluent which absorbed radiation at 280 nm was collected until the extinction fell to approx. one half of its maximum value (approx. 1 l). The bulk of the enzyme activity (63—85%) was eluted under these conditions while approx. 90% of the protein was retained by the column.

Stage 6. $(NH_4)_2SO_4$ was added to the stage 5 preparation (0.6 g/ml). After 60 min stirring, the precipitate was collected by centrifugation and suspended in 10 ml of 0.01 M Tris-HCl buffer, pH 7.9. The suspension was dialysed against several changes of the same buffer, and the small amount of residual insoluble material was removed by centrifuging and discarded. The clear supernatant was then applied to a 11 $\times$ 2.1 cm column of microgranular CM-cellulose which had been equilibrated with 0.01 M Tris-HCl buffer, pH 7.9. The column was washed with 90 ml of the same buffer and elution was then continued in this buffer with a linear NaCl concentration gradient (0—0.15 M NaCl, 40

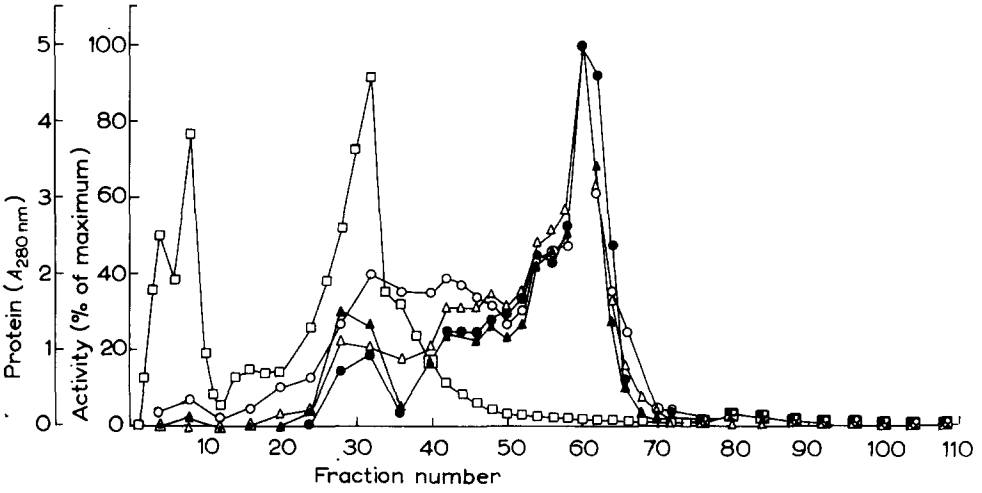

Fig. 1. Fractionation of stage 5 protein by chromatography on CM-cellulose showing the hydrolytic activity of fractions towards various substrates. Protein, □; adenosine 5′-phosphosulphate sulphohydrolase, ●; ATPase, △; pyrophosphatase, ▲; and phosphodiesterase, ○.

column volumes). Fractions (14 ml) were collected and assayed for protein and adenosine 5′-phosphosulphate sulphohydrolase activity and those of high specific activity (nos. 52—70, eluting in approx. 75 mM NaCl) were pooled. A typical elution profile is shown in Fig. 1.

Stage 7. The pooled fractions were concentrated to approximately 6 ml by ultrafiltration using an Amicon UM10 membrane. The concentrate was dialysed against 0.1 M acetic acid/NaOH (pH 5.5) containing 0.5 M NaCl. A small precipitate was removed by centrifuging and the supernatant was applied to a 3.5 X 0.5 cm column of 5′-AMP-Sepharose 4B which had been packed using 0.1 M acetic acid/NaOH buffer (pH 5.5) containing 0.5 M NaCl. After washing the column with 24 ml of the same buffer, highly purified enzyme was obtained by elution with the same buffer containing 5 mM AMP. Attempts to dialyse the enzyme at this stage against 0.01 M acetic acid/NaOH buffer (pH 5.2) resulted in serious loss of activity. Consequently, the enzyme was stored at −10°C in plastic vessels in 25-μl portions without further treatment. Although repeated freezing and thawing of enzyme solution led to considerable losses of enzyme activity, the frozen enzyme was stable for several months.

Results

Table I shows the progress of a typical enzyme purification. Three separate purifications produced final preparations with specific activities of 37.1, 37.3 and 42.2 units/mg. Although the overall recoveries were generally low (1—2%), these figures represent very high purifications (of the order of 10 000-fold) of the lysosomal-mitochondrial pellet.

SDS-polyacrylamide gel electrophoresis (7.5% gels) of the stage 7 material resulted in a single major band corresponding to a molecular weight of 51 000—53 000 for three different preparations. Heavy loading of gels revealed traces of an additional minor component (Fig. 2) moving ahead of the major band and corresponding to a molecular weight of 36 000—39 000.

Both acidic and basic polyacrylamide gel electrophoresis revealed single

TABLE I

SUMMARY OF THE PURIFICATION PROCEDURE FOR LYSOSOMAL ADENOSINE 5′-PHOSPHOSULPHATE SULPHOHYDROLASE FROM BOVINE LIVER

Stage	Procedure	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (munits/mg)	Recovery (%)	Purification
1	Preparation of mitochondrial-lysosomal suspension	7850	180 000	858	5.2	100	1.0
2	Freeze-thawing	7150	207 000	822	4.0	95	0.77
3	Centrifugation	6150	88 000	547	6.2	64	1.2
4	$(NH_4)_2SO_4$ fractionation	720	19 400	286	14.7	33	2.8
5	DEAE-cellulose	1050	2 730	243	89	28	17
6	CM-cellulose	208	39.5	84	2 116	9.8	407
7	Affinity chromatography on 5′-AMP Sepharose 4B	1.7	0.54	23	42 200	2.4	8115

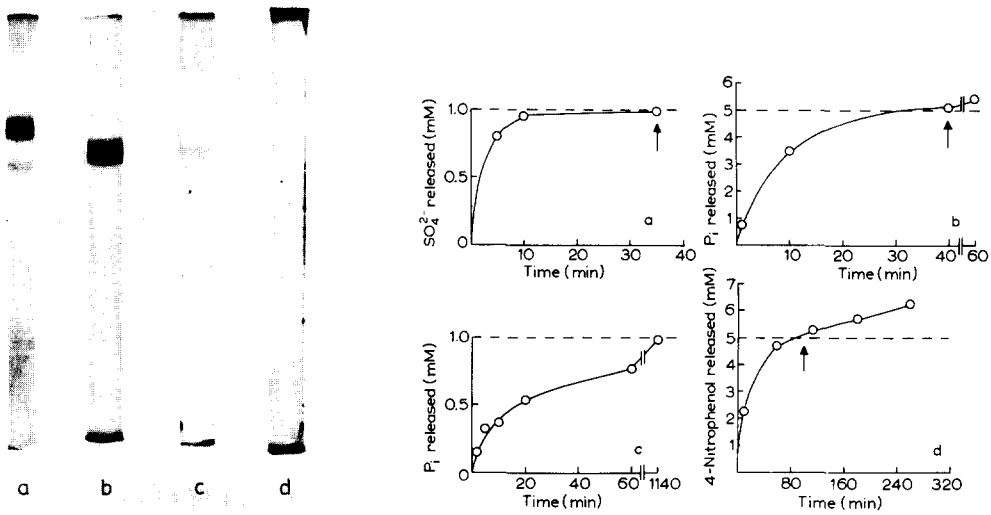

Fig. 2. Polyacrylamide gel electrophoresis of the stage 7 enzyme preparation. a, SDS polyacrylamide gel (16 μg protein); b, Acidic polyacrylamide gel (13 μg protein); c, As b except 2 μg protein; d, Basic polyacrylamide gel (5 μg protein).

Fig. 3. Typical reaction curves for hydrolysis catalysed by lysosomal adenosine 5′-phosphosulphate sulphohydrolase. a, Adenosine 5′-phosphosulphate. Incubation mixture: 1 mM adenosine 5′-phospho[^{35}S]-sulphate, 0.1 M acetic acid/NaOH buffer, pH 5.2, 0.1 ml 50% glycerol/enzyme in a total volume of 1.0 ml; 0.2 ml samples assayed. At 35 mins (arrow), 0.01 ml sample analysed for products by ion-exchange paper chromatography. b, ATP. Incubation mixture: 5 mM ATP, 0.10 M acetic acid/NaOH buffer, pH 5.8, 0.02 ml 50% glycerol/enzyme in a total volume of 0.8 ml. Samples (5 μl) were diluted to 0.2 ml with water and assayed. At 40 min (arrow), 0.5 μl sample was analysed by paper electrophoresis. c. Pyrophosphate. Incubation mixture: 0.5 mM pyrophosphate 0.1 M acetic acid/NaOH buffer, pH 5.8, and 0.01 ml of 50% glycerol/enzyme in a total volume of 0.4 ml. Samples (20 μl) were diluted to 0.2 ml with water and assayed. d. Bis(4-nitrophenyl)phosphate. Incubation mixture: 5 mM bis(4-nitrophenyl)phosphate, 0.05 M Tris-maleate/NaOH buffer, pH 6.8, and 0.05 ml of 50% glycerol/enzyme in a total volume of 0.08 ml. Samples (5 μl) were mixed with 1 ml 0.1 M NaOH and 4-nitrophenol measured in the usual way. At 100 mins (arrow), 0.05 ml sample was analysed by paper electrophoresis.

bands as shown in Fig. 2. Heavy loading on acidic gels failed to produce any additional bands. In all cases, the protein band corresponded with the sections of maximum adenosine 5′-phosphosulphate sulphohydrolase activity. The slow migration on basic gels (pH approx. 10 at the running temperature) indicates a high isoelectric point for the enzyme, in keeping with its behaviour on ion-exchange columns.

Substrate specificity

In view of the apparent homogeneity of the preparation on polyacrylamide gel electrophoresis, a study of the specificity of the purified enzyme towards a series of potential substrates was made. Relative activities towards various substrates are shown in Table II. No activity towards the sulphate esters, nitrocatechol sulphate and 4-nitrophenyl sulphate, was detected and 3′-phosphoadenosine 5′-phosphosulphate was attacked only very slowly. Several pyrophosphate compounds, however, were substrates: ADP, the phosphorus analogue of adenosine 5′-phosphosulphate, and FAD were relatively poor substrates whereas inorganic pyrophosphate (PP_i) was comparable with adenosine

TABLE II

ACTIVITY OF LYSOSOMAL ADENOSINE 5'-PHOSPHOSULPHATE SULPHOHYDROLASE TOWARDS VARIOUS COMPOUNDS AT pH 5.7

Substrate	Concentration (mM)	Specific activity (μmol substrate hydrolyzed/min per mg protein)
ATP	0.50	188
Adenosine 5'-phosphosulphate	0.50	44
PP_i	0.30	33
bis(4-nitrophenyl)Phosphate	5.00	14
FAD	0.02	3.9
ADP	5.00	3.5
3'-Phosphoadenosine 5'-phosphosulphate	0.02	0.07
4-Nitrophenyl-5'-phosphothymidine	0.05	0.05
4-Nitrophenyl phosphate *	5.00	0.12
O-Phospho-L-serine	0.10	0.10
O-Phospho-D-serine	0.10	0
DNA	1.5 **	0
RNA	1.5 **	0
5'-AMP	0.10	0
2'(3')-AMP	0.10	0
Nitrocatechol sulphate	5.00	0
4-Nitrophenyl sulphate	5.00	0
4-Nitrophenyl-3'-phosphothymidine	0.05	0
Glucose 6-phosphate	0.10	0

* Tris-maleate buffer, pH 6.8.
** mg/ml.

5'-phosphosulphate, and ATP was by far the best substrate of the compounds tested. There was virtually no activity towards other nucleotides and phosphomonoesters tested. However, some phosphodiesters were substrates, notably bis(4-nitrophenyl)phosphate and 4-nitrophenyl thymidine-5'-phosphate, but not the 3'-isomer, nor DNA or RNA.

In subsequent experiments, the four best substrates (ATP, adenosine 5'-phosphosulphate, PP_i and bis(4-nitrophenyl)phosphate) were chosen for further study. Typical reaction progress curves for these are shown in Fig. 3. In the final incubation mixtures, the release of SO_4^{2-}, P_i and 4-nitrophenol from adenosine 5'-phosphosulphate, ATP and bis(4-nitrophenyl) phosphate, respectively, had reached a 1 : 1 (and 2 : 1 in the case of P_i release from PP_i) stoichiometry with the amount of substrate originally present. At the points indicated by arrows, samples were analysed to identify the coproducts.

For adenosine 5'-phosphosulphate hydrolysis, a combination of ion-exchange paper chromatography and paper electrophoresis (pH 7.3) proved that the products were inorganic sulphate and 5'-AMP. No cyclic AMP or adenosine was detected. For bis(4-nitrophenyl)phosphate, the rapid release of only 1 mol of 4-nitrophenol per mol of bis(4-nitrophenyl)phosphate originally present (Fig. 3d) implies a true diesterase activity, i.e., hydrolysis of only one of the two ester bonds present. This was confirmed by paper electrophoresis (at pH 5.8) of the final incubation mixture which contained both 4-nitrophenyl phosphate and 4-nitrophenol, but very little bis(4-nitrophenyl)phosphate. The relatively slow increase in 4-nitrophenol production in the incubation mixture

after 100 min (Fig. 3d) is presumably due to the low activity of the enzyme towards 4-nitrophenyl phosphate (Table II). Similarly for ATP, paper electrophoretic analysis of the final incubation mixture revealed the presence of considerable amounts of AMP besides ADP. This presumably reflects the low activity of the enzyme towards ADP. The incubation was repeated using 0.01 mM [U-^{14}C]ATP (562 Ci/mol) and 1 μl 50% glycerol/enzyme per 0.2 ml incubation mixture. After 2 min incubation, paper electrophoresis followed by radioscanning of the dried electrophoretogram, revealed that the mixture contained 25% residual ATP, 68% ADP and only 7% AMP. This indicates that the primary product of ATP hydrolysis is indeed ADP, the reduced enzyme concentration and incubation time being now insufficient to allow further appreciable hydrolysis of ADP to AMP. After 10 min incubation, the mixture contained no detectable ATP and roughly equal amounts of ADP and AMP, showing the subsequent action of the enzyme on ADP.

pH vs. enzyme activity profiles

Fig. 4 shows the pH vs. enzyme activity profiles for the four substrates. For adenosine 5′-phosphosulphate, pyrophosphate and bis(4-nitrophenyl)phosphate, no discontinuities were observed when buffer type was changed, and smooth curves were obtained. For ATP however, acetic acid/NaOH buffers in the range pH 3.9—6.1 produced higher activities than 3,3-dimethylglutaric acid/NaOH or Tris-maleate/NaOH buffers in the same range. Adenosine 5′-phosphosulphate and bis(4-nitrophenyl)phosphate have quite broad pH profiles with optima at pH 6 and 7, respectively. ATP and pyrophosphate show sharper profiles with maxima at slightly lower pH in the 5—6 range.

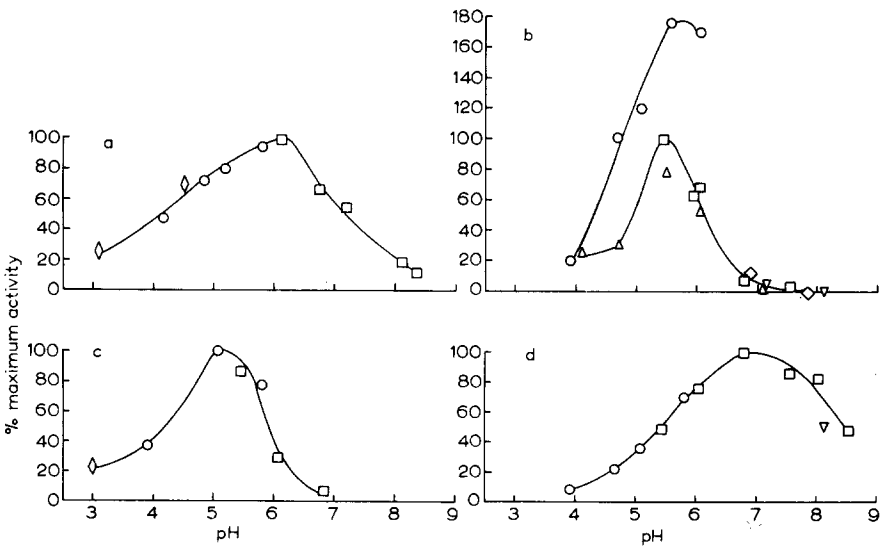

Fig. 4. pH-activity profiles for the hydrolysis of various substrates by purified lysosomal adenosine 5′-phosphosulphate sulphohydrolase. The substrates used were (a) adenosine 5′-phosphosulphate, 1.0 mM; (b) ATP, 0.5 mM; (c) pyrophosphate 0.3 mM; (d) bis(4-nitrophenyl)phosphate, 5.0 mM. The following buffers were used: ○, 0.1 M acetic acid/NaOH; □, 0.1 M Tris-maleate/NaOH; △, 0.1 M 3,3-dimethylglutaric acid/NaOH; ◇, 0.1 M formic acid/NaOH; ▽, 0.1 M glycylglycine/NaOH. Results in b were calculated as a percentage of the activity found in Tris/maleate (pH 5.4).

Kinetic parameters

All four activities exhibit simple Michaelis-Menten behaviour, with ATP and pyrophosphate showing substrate inhibition at higher concentrations. Values of K_m and V for each substrate at pH 5.7 were obtained from double reciprocal plots in the usual way. The rate constant, k_{cat} for the slowest step in the breakdown of enzyme-substrate complex for each substrate was calculated by dividing V by the enzyme concentration employed in each set of experiments. Enzyme concentrations were calculated assuming homogeneity of the protein and a molecular weight of 53 000 (see above). The results are shown in Table III. On the basis of K_m, adenosine 5′-phosphosulphate is the 'best' substrate for the enzyme whereas bis(4-nitrophenyl)phosphate has a relatively high K_m. The pH used in these experiments (5.7) is near the pH optima for adenosine 5′-phosphosulphate, ATP and PP_i (see Fig. 4) but rather more remote from that for bis(4-nitrophenyl)phosphate, a possible reason for the latter's high K_m value. However, measurement of K_m for bis(nitrophenyl)phosphate at its pH optimum (6.8 in Tris-maleate buffer) revealed an even higher K_m of 4.9 mM.

Evidence for a single protein

The detection of four significant hydrolytic activities raises the possibility that they may be associated with more than one protein. The following evidence suggests that this is not the case and that all the activities reside in a single protein.

a. Ion-exchange chromatography. Details of the chromatography of the stage 5 material on a CM-cellulose ion-exchange column have already been described above. In this experiment, the bulk of the adenosine 5′-phosphosulphate sulphohydrolase activity was eluted at a relatively late stage with a characteristic and reproducible elution profile (Fig. 1). This finding presents a test for the common identity of the four enzyme activities, because if they do correspond to a single protein then the elution profiles should be the same for all four. Fractions from such an elution were therefore assayed for each of the four activities. The results are shown in Fig. 1 in which the enzyme activity curves have been normalized by calculating, in each case, the activities as a percentage of the maximum value found. Although in the early stages, the activity curves are quite divergent, in the later stages of elution (fractions 52—70) from which the final stage 7 preparation was ultimately derived, they

TABLE III

VALUES OF K_m AND k_{cat} FOR THE ENZYMATIC HYDROLYSIS OF VARIOUS SUBSTRATES BY LYSOSOMAL ADENOSINE 5′-PHOSPHOSULPHATE SULPHOHYDROLASE AT pH 5.7

Experimental details are in the text.

Substrate	K_m (mM)	k_{cat} (min^{-1})
Adenosine 5′-phosphosulphate	0.04	2 670
ATP	0.14	11 700
PP_i	0.14	5 400
bis(4-nitrophenyl)Phosphate	0.40	1 040

are virtually identical. Activity ratios for fractions taken across the peak are constant with a standard deviation of 20%. This shows that the four enzyme activities are associated with either a single protein, or two or more proteins of very similar ionic properties.

b. BioGel P-150 gel filtration chromatography. An attempt was made to separate the four activities on the basis of molecular size. A sample (1 ml) of the pooled material from stage 6 was eluted from a column (80 × 2 cm) of BioGel P-150 gel filtration medium with 0.05 M acetic acid/NaOH (pH 5.2) buffer. Eluant fractions (1 ml) were assayed for hydrolytic activity towards adenosine 5′-phosphosulphate, ATP, PP_i and bis(4-nitrophenyl)phosphate. The results are shown in Fig. 5 in which the curves have again been normalized. Clearly, the activity peaks for the four activities are coincident. Following calibration of the column with standard proteins, the molecular weight of the protein associated with the enzyme activities was found to be 56 000. This result is in good agreement with the value determined by sodium dodecyl sulphate gel electrophoresis for the major component of the final stage 7 preparation (52 000 ± 1000), and furthermore it shows that none of the four activities is associated with the minor contaminant (molecular weight 37 000—39 000) visible on overloaded SDS polyacrylamide gels. The activity ratios across the peak again remained constant with a standard deviation of 20%.

c. Acid and basic polyacrylamide gel electrophoresis. When acidic polyacrylamide gels were assayed or stained for each of the four activities, single bands were obtained which were coincident with each other and with the single protein band. On basic gels, migration was very slow (1—3% of the tracker dye) but in this case also, adenosine 5′-phosphosulphate sulphohydrolase and phosphodiesterase both coincided with the protein band. Attempts to detect ATPase and pyrophosphatase were unsuccessful, presumably because of the sharp cut-off in activity towards these substrates above pH 7 (Fig. 4).

d. Inhibition studies. ATP, adenosine 5′-phosphosulphate and PP_i were tested separately as inhibitors of the activity of the final stage 7 enzyme towards bis(4-nitrophenyl)phosphate. In each case, the double reciprocal plots [17] were linear, with a common intercept on the $1/v$ axis, indicating strictly

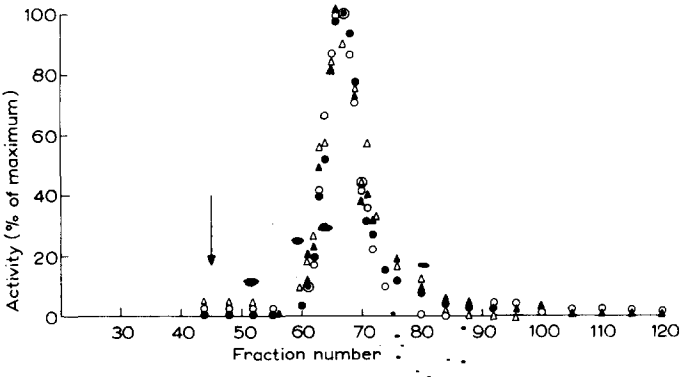

Fig. 5. Fractionation of stage 6 protein by gel filtration chromatography on BioGel P-150. Adenosine 5′-phosphosulphate sulphohydrolase, ●; ATPase, △; pyrophosphatase, ▲; and phosphodiesterase, ○. The arrow indicates the void volume.

TABLE IV

INHIBITION CONSTANTS FOR THE INHIBITION OF PHOSPHODIESTERASE ACTIVITY BY ADENOSINE 5'-PHOSPHOSULPHATE, ATP AND PP_i

Phosphodiesterase activity was assayed in the normal way at pH 5.7, and included in the table are the K_m values for the inhibitors under the same conditions.

Compound	K_m (mM)	Inhibition of phosphodiesterase activity	
		Type	K_i (mM)
Adenosine 5'-phosphosulphate	0.04	Competitive	0.08
ATP	0.14	Competitive	0.10
PP_i	0.14	Competitive	0.14

competitive inhibition. Secondary plots of apparent K_m against inhibitor concentration were also linear, yielding the K_i values shown in Table IV. The K_i values are in close agreement with the K_m values for ATP, adenosine 5'-phosphosulphate and PP_i at this pH (5.7).

Discussion

Bovine liver lysosomal adenosine 5'-phosphosulphate sulphohydrolase has now been purified (10 000-fold) to apparent homogeneity on the basis of acidic, basic and sodium dodecyl sulphate polyacrylamide gel electrophoresis. The protein has a molecular weight of 52 000 by SDS polyacrylamide gel electrophoresis and 56 000 by gel filtration, indicating the absence of subunits. In these respects, it resembles the bovine liver cytosol enzyme. However, the lysosomal enzyme is fairly basic in character (pI = 8—10) as judged by its behaviour on ion-exchange columns and polyacrylamide gel electrophoresis whereas the cytosol enzyme is relatively much more acidic since it is still retarded on DEAE-cellulose columns at pH 6.6. Furthermore, the ox liver cytosol enzyme has a sharp pH optimum, whereas the lysosomal enzyme shows a broad pH profile (Fig. 4), a property shared with the rat liver system [4]. Substrate specificity studies show that the bovine lysosomal enzyme will hydrolyse a range of phosphosulphate, pyrophosphate and phosphodiester substrates (Table II). In contrast, the cytosol enzyme is highly specific for adenosine 5'-phosphosulphate, although its K_m (0.95 mM) is more than 20 times greater. Any doubts that the lysosomal activities may be due to the presence of more than one enzyme are allayed by the strong collective evidence of identical elution patterns on CM-cellulose ion-exchange and BioGel P-150 gel filtration chromatography, coincidence of all activities with a single protein band on acidic and basic polyacrylamide gel electrophoresis, competitive inhibition of bis(4-nitrophenyl)phosphate hydrolysis by the other substrates adenosine 5'-phosphosulphate, ATP and PP_i, with inhibition constants closely similar to their respective K_m values. In short, the bovine liver lysosomal adenosine 5'-phosphosulphate sulphohydrolase is quite distinct from the cytosol enzyme, and in some respects resembles its counterpart in rat-liver lysosomes. It is a simple hydrolase, liberating AMP and SO_4^{2-}, and is not an

adenosine 5′-phosphosulphate cyclase of the type found [18] in *Chlorella pyrenoidosa* which produces cyclic AMP and SO_4^{2-}.

The broad substrate specificity of the lysosomal enzyme, besides emphasising its dissimilarity to the cytosol enzyme, more importantly sheds some light on its physiological role. Since the enzymes responsible for the synthesis of adenosine 5′-phosphosulphate and its conversion to 3′-phosphoadenosine 5′-phosphosulphate are located in the soluble fraction, it seems reasonable to suppose that the cytosol adenosine 5′-phosphosulphate sulphohydrolase, in conjuction with these synthetic enzymes and other hydrolytic enzymes [3] may well play a role in the control of sulphate conjugation (e.g. of phenols, steroids, acid glycosaminoglycans). However, the role of lysosomal adenosine 5′-phosphosulphate sulphohydrolase is much less easy to comprehend since it is generally accepted that lysosomes are concerned mainly with the intracellular digestion of macromolecular species. The present discovery of additional activity towards bis(4-nitrophenyl)phosphate and 4-nitrophenyl-5′-phosphothymidine, but not the 3′-isomer, at first implied nuclease activity, since many nuclease enzymes hydrolyse these chromogenic substrates [19,20]. However, no such nuclease activity could be detected towards either DNA or RNA.

Brightwell, Tappel and co-workers in a series of papers reported the presence of a hydrolase in rat-liver lysosomes with multiple activity similar to adenosine 5′-phosphosulphate sulphohydrolase. An acid pyrophosphatase in lysosomes was found to hydrolyse both pure FAD and FAD present in whole mitochondria [21]. This enzyme was identified [22] with one of the phosphodiesterases in rat liver, namely the phosphodiesterase IV acting on bis(4-nitrophenyl)phosphate, which was also suspected of activity towards 4-nitrophenyl-5′-phosphothymidine (phosphodiesterase I), a suspicion recently confirmed [23]. The enzyme's specificity was then extended [24] to include ATP and PP_i among several other pyrophosphates, but not ADP. Activity towards oligo- or polynucleotides was not investigated. Human liver has been less extensively studied [25], but there also a lysosomal pyrophosphatase/phosphodiesterase is present with activity towards bis(4-nitrophenyl)phosphate, ATP and PP_i, but not ADP. The present work confirms this pattern and extends it to include adenosine 5′-phosphosulphate using, for the first time, a highly purified enzyme preparation from bovine liver lysosomes.

Clearly then, the lysosomal adenosine 5′-phosphosulphate sulphohydrolase is a much less specific enzyme than its cytosol counterpart and although it is not possible to be precise about the physiological role of the former, it now seems unlikely that the enzyme is restricted in vivo to adenosine 5′-phosphosulphate hydrolysis. The substrates for which activity has been found in this work are all, strickly speaking, acid anhydrides. Thus, the bonds cleaved in ATP, PP_i, ADP and FAD are anhydride links between two phosphoric acid residues; adenosine 5′-phosphosulphate is cleaved at a mixed anhydride link between sulphuric acid and a phosphoric acid residue; even the chromogenic substrates (bis(4-nitrophenyl)phosphate, 4-nitrophenyl phosphate, 4-nitrophenyl-5′-phosphothymidine) should properly be considered [26] not as esters, but as mixed anhydrides of a phosphoric acid with the weak acid, 4-nitrophenol. The pattern begins to emerge, therefore, of an enzyme hydrolysing acid anhydrides at least

one acid residue of which is always phosphoric acid (or a derivative thereof). Secondary lysosomes and autophagosomes may well contain an abundance of such anhydrides, depending on the material undergoing digestion. For example, digestion of mitochondria by lysosomes has been shown to occur both in vivo [27] and in vitro [28]. Autophagy of mitochondria in vivo would thus presumably result in the release of such compounds as FAD, ATP and ADP within secondary lysosomes and the 'acid anhydride hydrolase' could then usefully function in a scavenging role to recover the nucleoside monophosphates.

Acknowledgements

We wish to thank the Wellcome Trust for financial support. K.M.R. is grateful to the Leverhulme Trust for a Research fellowship.

References

1 Robbins, P.W. and Lipman, F. (1958) J. Biol. Chem. 233, 681—685
2 Levi, A.S. and Wolf, G. (1969) Biochim. Biophys. Acta 178, 262—282
3 Denner, W.H.B., Stokes, A.M., Rose, F.A. and Dodgson, K.S. (1973) Biochim. Biophys. Acta 315, 394—401
4 Bailey-Wood, R., Dodgson, K.S. and Rose, F.A. (1970) Biochim. Biophys. Acta 220, 284—299
5 Stokes, A.M., Denner, W.H.B., Rose, F.A. and Dodgson, K.S. (1973) Biochim. Biophys. Acta 302, 64—72
6 Stokes, A.M., Denner, W.H.B. and Dodgson, K.S. (1973) Biochim. Biophys. Acta 315, 402—411
7 Cherniak, R. and Davidson, E.A. (1964) J. Biol. Chem. 239, 2986—2990
8 Mattock, P. (1967) Ph.D. Thesis, University of Wales
9 Bannerjee, R.K. and Roy, A.B. (1966) Mol. Pharmacol. 2, 56—63
10 Parvin, R. and Smith, R.A. (1969) Anal. Biochem. 27, 65—72
11 De Duve, C., Pressman, B.C., Gianetto, R., Wattiaux, R. and Appelmans, F. (1955) Biochem. J. 60, 604—617
12 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
13 Denner, W.H.B. and Stokes, A.M. (1972) J. Chromatogr. 69, 426—427
14 Weber, K., Pringle, J.R. and Osborn, M. (1972) Methods Enzymol. 26, 3—27
15 Gabriel, O. (1971) Methods Enzymol. 22, 565—578
16 Dixon, M. (1953) Biochem. J. 54, 457—458
17 Lineweaver, H. and Burk, D. (1934) J. Am. Chem. Soc. 56, 658—663
18 Lik-shing Tsang, M. and Schiff, J.A. (1976) Eur. J. Biochem. 65, 113—121
19 Laskowski, M. (1971) in The Enzymes (Boyer, P.D., ed.), Vol. IV, pp. 313—328, Academic Press, New York
20 Bernardi, A. and Bernardi, G. (1971) in The Enzymes (Boyer, P.D., ed.), Vol. IV, pp. 329—336, Academic Press, New York
21 Ragab, M.H., Brightwell, R. and Tappel, A.L. (1968) Arch. Biochem. Biophys. 123, 179—185
22 Brightwell, R. and Tappel, A.L. (1968) Arch. Biochem. Biophys. 124, 325—332
23 Erecinska, M., Sierakowska, H. and Shugar, D. (1969) Eur. J. Biochem. 11, 465—471
24 Brightwell, R. and Tappel, A.L. (1968) Arch. Biochem. Biophys. 124, 333—343
25 Callahan, J.W., Lassila, E.L. and Phillippart, M. (1974) Biochem. Med. 11, 250—261
26 Spahr, P.F. and Gesteland, R.F. (1970) Eur. J. Biochem. 12, 270—284
27 De Duve, C. and Wattiaux, R. (1966) Annu. Rev. Physiol. 28, 435—492
28 Savant, P.L., Desai, I.D. and Tappel, A.L. (1964) Biochim. Biophys. Acta 85, 93—102