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Purification and Properties of Arylsulphatase B of Human Liver

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1. A purification scheme for an arylsulphatase B from human liver is described. Specificity of purification was achieved by the use of affinity chromatography on an agarose–4-hydroxy-2-nitrophenyl sulphate derivative. The scheme provides a rapid and convenient method for preparation of a highly purified enzyme. 2. The purified enzyme was examined by isoelectric focusing electrophoresis on polyacrylamide gel and by ultracentrifugation and was found to be catalytically homogeneous, with an apparent molecular weight of 50000 and a specific activity of 93.3 units/mg of protein. 3. The kinetic properties of the purified preparation and the effect of various amino acid group-specific reagents on the catalysis of the enzyme are described. The involvement of histidine residues in the active site of the enzyme is suggested. 4. The purified enzyme lost activity rapidly on freezing. The implication of this observation is discussed in terms of a possible dissociation–reaggregation phenomenon induced by cold treatment.

Interest in the lysosomal arylsulphatase enzymes (arylsulphate sulphohydrolase, EC 3.1.6.1) has increased as these enzymes have been implicated in a number of pathological conditions in man. The accumulation of cerebroside sulphate in tissues of patients suffering from metachromatic leukodystrophy was shown to be paralleled by a large decrease in the activity of arylsulphatase A (Mehl & Jatzkewitz, 1964; Austin *et al.*, 1965; Mehl & Jatzkewitz, 1968). This physiological role for arylsulphatase A was confirmed by the proof of the identity of the cerebroside sulphatase and arylsulphatase A enzymes (Porter *et al.*, 1972; Farooqui & Bachhawat, 1973; Bleszynski & Roy, 1973). Diagnostic use of arylsulphatase A activity has been made in cases of different forms of leukodystrophy and other lysosomal disorders (Thomas & Howell, 1972).

Clear circumstantial evidence exists for the association of arylsulphatase B with the metabolism of sulphated mucopolysaccharides. Stumpf *et al.* (1973) reported a gross deficit in the activity of this enzyme in the liver, kidney and spleen of patients with the Maroteaux-Lamy syndrome, a congenital mucopolysaccharidosis characterized by excessive accumulation of dermatan sulphate. A similar deficiency in the activity of arylsulphatase B has been reported by Fluharty *et al.* (1974) in cultured fibroblasts derived from patients with the Maroteaux-Lamy syndrome. It is also possible that the 'Hunter corrective factor', which degrades sulphated mucopolysaccharides and which was isolated from human urine by Bach *et al.* (1973), is an arylsulphatase B. All of these studies suffer from the defect that partially purified and inadequately characterized preparations of arylsulphatase B were used. Also, the assay method used to measure arylsulphatase B in the presence of arylsulphatase A (Baum *et al.*, 1959) has severe limitations and its use has been criticized by Worwood *et al.* (1973). The precise determination of the independent activities of the two enzymes requires prior separation of the enzymes.

Although highly purified preparations of arylsulphatase B have been made from many mammalian tissues (Allen & Roy, 1968; Nicholls & Roy, 1971; Bleszynski & Roy, 1973), preparations from human tissues have not been so well characterized. Dodgson & Wynn (1958) described the preparation of a partially purified enzyme from human liver; Balasubramanian & Bachhawat (1963) and Harinath & Robins (1971) described the purification of arylsulphatase B of human brain by ion-exchange chromatography. These preparations were not homogeneous and were not completely characterized. The present paper describes the development of a rapid method of purification to homogeneity of an arylsulphatase B of human liver by using the affinity matrix reported in a preliminary communication (Agogbua & Wynn, 1975). The kinetic properties of this enzyme are described, together with the effect of certain group-specific amino acid reagents giving an indication of the nature of the active site.

Materials and Methods

Substrates

Dipotassium 2-hydroxy-5-nitrophenyl sulphate (4-nitrocatechol sulphate) was prepared by the

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method of Roy (1953) as modified by Dodgson & Spencer (1956). Potassium *p*-nitrophenyl sulphate was prepared by the method of Burkhardt & Lapworth (1926) as modified by Dodgson & Spencer (1953). The monopotassium salts of 4-hydroxy-2-nitrophenyl sulphate and 4-hydroxy-3-nitrophenyl sulphate were prepared by the method of Smith (1951).

Other reagents

Sephadex G-100 and Sepharose 4B were obtained from Pharmacia, Uppsala, Sweden; CM-cellulose (CM 52) was from Whatman, Reeve Angel, London EC4V 6AY, U.K. Ampholine was obtained from LKB Produkter AB, Bromma 1, Sweden. Rose Bengal and *p*-nitrobenzyl azide were supplied by Eastman Organic Chemicals, New York, N.Y., U.S.A. Pyridoxal 5-phosphate and *N*-acetylimidazole were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Other reagents were of analytical grade. Common laboratory reagents were redistilled or recrystallized from appropriate solvents before use.

Assay procedure

The activity of arylsulphatase B was measured by a modification of the method of Dodgson *et al.* (1953). Unless otherwise stated incubation was for 1h at 37°C. To 0.5ml of enzyme solution, adjusted to pH6.1 and preincubated at 37°C for 3min, was added 0.5ml of 20mM-4-nitrocatechol sulphate in 0.25M-sodium acetate adjusted to pH6.1 with 0.25M-acetic acid, similarly preincubated at 37°C. The reaction was terminated by the addition of 4.0ml of 0.2M-NaOH and the liberated 4-nitrocatechol was measured spectrophotometrically at 515nm ($\varepsilon_{515} = 10900$) against a blank, prepared by incubating enzyme and substrate separately and mixing after the addition of the NaOH to the enzyme solution. Enzyme solutions were diluted so that the absorbance of the liberated 4-nitrocatechol did not exceed 0.8.

One unit of enzyme activity is defined as the amount of enzyme which will liberate 1μmol of 4-nitrocatechol/min under the above experimental conditions.

Protein determination

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Experimental and Results

Preparation of soluble enzyme extract

Fresh non-pathological liver was obtained from the autopsy room of Manchester Royal Infirmary, usually within 48h of death. After removal of vessels and encapsulating tissue, the liver was cut into approx. 100g pieces. Each piece was diced and homogenized in 400ml of 0.1M-sodium acetate/acetic acid buffer, pH4.5, at 4°C in a Waring blender. The homogenates were pooled and left at 4°C for 15min before centrifugation at 23000*g* for 30min. After removal of the supernatant, the debris was washed by suspension in 4vol. of buffer followed by centrifugation as before. The two supernatants were combined to form the soluble enzyme extract.

Isoelectric focusing

This procedure was carried out according to the manufacturer's instructions, by using an LKB 8101 column (110ml capacity) filled with wide-range (pH3–10) Ampholine. The pH gradient was stabilized by the superimposition of a sucrose gradient. Enzyme samples were dialysed extensively against water and suitably concentrated by ultrafiltration through a Diaflo PM 10 membrane (Amicon Corp., High Wycombe, Bucks., U.K.) before introduction into the middle of the gradient. Separations took 56h at 4°C. The finishing voltage was 400V at about 0.5mA current. Fractions (2ml) were collected, the pH of each was measured and then adjusted to pH5.0 by the addition of 1.0M-sodium acetate/acetic acid buffer, pH5.0, before assay for arylsulphatase activity and protein.

The crude soluble enzyme extract showed two peaks of arylsulphatase activity with pI 4.3 and 7.5. Comparison of these values with those found for the ox liver enzymes (Allen & Roy, 1968) suggested that the activities corresponded to arylsulphatases A and B respectively, and this was confirmed by examination of the pH profile of the two fractions.

Purification procedure (*Table* 1)

Stage 1: *batch separation on CM* 52 *CM-cellulose.* The results of isoelectric focusing suggested that rapid and simple separation of arylsulphatases A and B might be achieved by adsorption on ion-exchange cellulose at pH4.5, under which conditions arylsulphatase B should be adsorbed strongly, leaving arylsulphatase A in solution.

CM-cellulose (CM 52, pre-swollen, microgranular; 120g) was equilibrated and washed several times in 0.1M-sodium acetate/acetic acid buffer, pH4.5, to remove the fines. The washed resin was stirred into the soluble enzyme extract derived from 1kg of liver and the mixture stirred at 4°C for 30min. Centrifugation at 1000*g* for 10min at 4°C gave a straw-coloured supernatant containing mainly arylsulphatase A. The resin was washed with 4vol. of cold buffer through a sintered funnel of coarse mesh. For elution of arylsulphatase B, the resin was stirred in 400ml of 1.0M-

Table 1. *Purification scheme for an arylsulphatase B from human liver*

Extraction and details of each step are as described in the main text. The starting material was 1.5kg of fresh liver. One unit of activity is defined as amount of enzyme which releases 1 μmol of nitrocatechol/min.

Fraction	Total activity (units)	Total protein (mg)	Specific activity (units/mg of protein)	Yield (%)
Total soluble extract (A+B)	12000	125000	0.096	100
Batch CM 52-adsorbed fraction (sulphatase B)	8650	42000	0.21	72
Dialysed 40–60%-satd.-$(NH_4)_2SO_4$ ppt.	10260	18000	0.57	84
CM 52 column eluate	4500	525	8.6	37
Affinity chromatography	4330	46	93.3	36

sodium acetate/acetic acid buffer, pH6.1, for 20min. The supernatant from centrifugation at 1000*g* for 10min contained the bulk of the arylsulphatase B activity, but to gain maximum yield the elution and centrifugation procedure was repeated and the supernatants were combined.

Stage 2: *$(NH_4)_2SO_4$ precipitation.* Solid $(NH_4)_2SO_4$ was added to the combined eluates from Stage 1 until the solution was 60% saturated. After stirring at 4°C for 1h, the precipitated protein was obtained by centrifugation at 72000*g* for 20min and then suspended by stirring into 50ml of a 40%-saturated $(NH_4)_2SO_4$ solution for 30min. The suspension was clarified by centrifugation at 72000*g* for 20min and the supernatant, containing all the arylsulphatase B activity, was dialysed against running tap water for 4h at 4°C and then against three changes of 4 litres of 0.1M-sodium acetate/acetic acid buffer, pH4.5. Protein which was precipitated on dialysis was removed by centrifugation. This procedure resulted in a twofold increase in specific activity and a slight increase in total activity, probably owing to the removal of endogenous inhibitors.

Stage 3: *ion-exchange chromatography on CM-cellulose.* The diffusate from Stage 2 was concentrated by ultrafiltration through a Diaflo PM 10 membrane to about 50ml and applied to a column (78cm× 2.5cm) of CM-cellulose (CM 52) previously equilibrated with 0.1M-sodium acetate/acetic acid buffer, pH4.5. The column was eluted with a pH gradient (4.5–6.1) superimposed on a linear salt gradient from 0.1 to 1.0M-sodium acetate at a flow rate of 20ml/h. The effluent was continuously monitored at 280nm and the 10ml fractions collected were assayed for protein and arylsulphatase B activity. The enzyme was eluted as a single peak (Fig. 1) well separated from the major protein peaks. A minor peak of unadsorbed activity is probably the result of contamination by arylsulphatase A. Fractions containing more than 50% of the maximum were pooled and assayed for protein and arylsulphatase B activity. The pooled fractions represented about 40% recovery of the applied activity and showed a 15-fold purification in this stage.

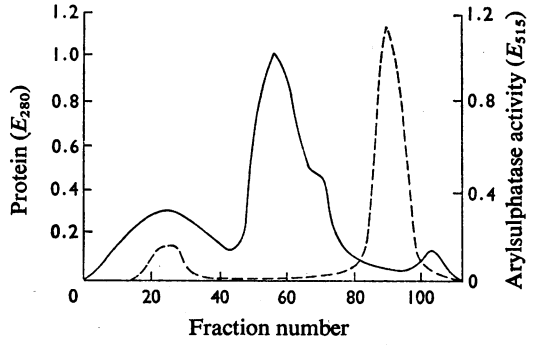

Fig. 1. *Ion-exchange chromatography on CM-cellulose of 40–60%-satd. $(NH_4)_2SO_4$ fraction of human liver extract*

Elution was by a combined salt and pH gradient as described in the text. Arylsulphatase B activity (----) was determined spectrophotometrically with 4-nitrocatechol sulphate as substrate. Protein (——) was measured by extinction at 280nm.

Stage 4: *affinity chromatography.* The highly specific affinity matrix previously described (Agogbua & Wynn, 1975) was used to adsorb selectively the arylsulphatase B from the Stage 3 preparation. This matrix was prepared from the *p*-aminobenzamidoethyl derivative of Sepharose 4B (Cuatrecasas, 1970) by coupling with 4-hydroxy-2-nitrophenyl sulphate after diazotization.

The enzyme solution, after dialysis against 0.1M-sodium acetate/acetic acid buffer, pH6.1, and concentration by ultrafiltration to 30ml containing 2.5mg of protein/ml, was applied to a column (8cm×1cm) of affinity matrix previously equilibrated with the same buffer. The column was washed with 80ml of starting buffer and the arylsulphatase B eluted with 60ml of buffer to which NaCl (0.5M final concentration) had been added. The two major peaks of unadsorbed protein showed no arylsulphatase B activity and this enzyme was selectively eluted at the higher ionic strength. The pooled fractions containing

enzyme activity were assayed for protein and arylsulphatase activity after exhaustive dialysis against 0.25M-sodium acetate/acetic acid buffer, pH6.1. Over 95% of the applied activity was recovered in this stage and the final preparation had a specific activity of 93.3 units/mg of protein.

The results of the overall purification procedure (Table 1) show that 36% of the activity of the crude soluble extract was recovered (this value disregards the contribution of the arylsulphatase A to the initial activity) and that approximately 1000-fold purification was achieved.

Physical and kinetic properties of purified enzyme

Various physical and kinetic parameters measured by conventional techniques are given in Table 2. Sedimentation-velocity studies showed a single homogeneous peak.

Electrophoretic characterization

A modified method of Dubois & Baumann (1973) was used. The gel solution was 7.5g of acrylamide, 0.1g of *NN'*-methylenebisacrylamide, 0.85mg of riboflavin and 0.2ml of tetramethylethylenediamine in 100ml of 0.4M-sodium acetate/acetic acid buffer, pH3.0. Gels were cast in tubes (7cm×0.8cm) by photopolymerization for 30min. Anode and cathode reservoirs were filled with 1.0M-sodium acetate/acetic acid buffer, pH3.0. Enzyme solution (100μl at 0.5mg/ml) was loaded with a trace of sucrose to minimize diffusion. Separation was for 90min at a current of 4mA/gel and the tracking dye was 0.04% Methyl Green.

Arylsulphatase B activity was detected by treatment of extruded gels with 0.5M-sodium acetate/acetic acid buffer, pH6.1, for 15min, 20mM-4-nitrocatechol sulphate in the same buffer for 20min and finally 0.2M-NaOH. Protein was detected by using the Coomassie Blue method of Malik & Berie (1972). Arylsulphatase B activity of the purified enzyme was coincident with the single protein band detected, indicating the electrophoretic homogeneity of the preparation.

Isoelectric focusing of the purified preparation with ampholyte of pH range 5–8 similarly showed a single coincident band of protein and enzyme activity.

Table 2. *Physical and kinetic properties of purified arylsulphatase B from human liver*

Molecular weight by gel filtration	47000–54000
Molecular weight by sedimentation equilibrium	59000–61000
pH optimum (nitrophenol sulphate as substrate)	6.1
Optimum substrate concentration (4-nitrocatechol sulphate as substrate)	10mM
Michaelis constant (K_m)	0.8mM
$s_{20,w}$	2.5S
$E^{1\%}_{280}$	0.98

Table 3. *Effect of bivalent cations and other compounds on the activity of arylsulphatase B*

The compounds were included in the reaction mixture at the final concentrations shown. Arylsulphatase B activity was determined as described in the text.

Compound	Concentration (M)	Activity (% of control)
$BaCl_2$	0.025	59
$CaCl_2$	0.01	53
$CuSO_4$	0.01	3
$MgCl_2$	0.01	60
$MnCl_2$	0.01	84
EDTA	0.01	160

Effect of bivalent cations

Various cations had an inhibitory effect when included in the enzyme assay reaction mixture (Table 3). This effect was most marked with Cu^{2+} and appeared to be relatively non-specific. EDTA at a concentration of 10mM gave a 60% activation, suggesting that inhibitory cations were normally present in the reaction mixture.

Effect of group-specific reagents

(*a*) *Thiol reagents.* Purified enzyme treated with either 2mM-*p*-chloromercuribenzoate or -*N*-ethylmaleimide for 1h at 20°C showed no inhibition of enzyme activity. Treatment with dithiothreitol (2mM) gave a 20% increase in activity, probably owing to the removal of inhibitory metal ions.

(*b*) *Tyrosine reagents.* Arylsulphatase B activity was not inhibited by *N*-acetylimidazole at a concentration of 4mM. This lack of inhibition by *p*-chloromercuribenzoate, *N*-ethylmaleimide and *N*-acetylimidazole indirectly rules out the possibility of amino-group involvement in the active site. This was confirmed by the fact that both fluorodinitrobenzene and trinitrobenzenesulphonic acid were without effect on the activity.

Effect of iodoacetate

When the enzyme solution (0.12mg/ml) was treated with iodoacetate at 40°C for 1h before assay of arylsulphatase B activity, concentration-dependent inhibition was observed (Fig. 2), the activity being completely abolished at 10mM-iodoacetate. This

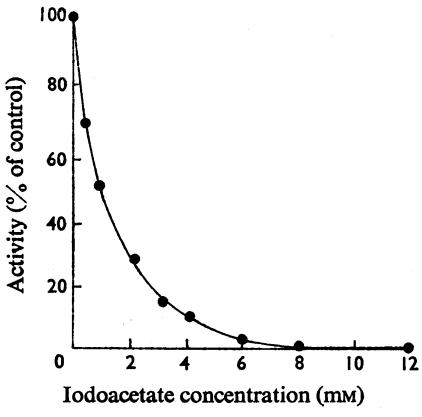

Fig. 2. *Effect of various concentrations of iodoacetate on the activity of purified arylsulphatase B*

The enzyme was incubated at 40°C for 1 h with iodoacetate before assay of activity. The activity is expressed as a percentage of that of an untreated control.

inhibition was decreased to about 28% when incubation with 10mM-iodoacetate was in the presence of 30mM-nitrocatechol sulphate. These results taken together with the effect of group-specific reagents indicate an essential histidine residue.

Photo-oxidation

When purified arylsulphatase B was subjected to photosensitized oxidation in the presence of 1mM-Rose Bengal by the method of Westhead (1965), the enzyme lost over 80% of its activity. However, considerable precipitation occurred during the photo-oxidation and no firm conclusion could be drawn from this experiment about the involvement of histidine residues. The inactivation may have resulted from a change in conformation induced by photo-sensitization.

When the experiment was repeated in the presence of 1mM-pyridoxal 5-phosphate, the inactivation was 55%, but in this case the inactivation was stopped completely by the inclusion of 30mM-4-nitrocatechol sulphate in the reaction mixture. Previous workers (Rippa & Pontremoli, 1969; Davis *et al.*, 1970) have shown that histidine residues in proteins may be destroyed by photo-oxidation in the presence of pyridoxal 5-phosphate.

Cold instability of the purified enzyme

During the preparation and purification of the arylsulphatase B from human liver, it was observed that solutions of the enzyme in 0.1M-sodium acetate/acetic acid buffer, pH 6.1, rapidly lost activity when stored frozen. In most cases, the activity fell to less than 30% of the original. This phenomenon has been previously reported (Raijman & Grisolia, 1961; Havir *et al.*, 1965; Irias *et al.*, 1969), although the physicochemical basis of such cold lability in enzymes in general is not clearly understood.

A preliminary investigation of cold inactivation of arylsulphatase B was undertaken along the lines suggested by Irias *et al.* (1969) in their work on the cold inactivation of pyruvate carboxylase.

Samples (0.5ml) of the pure enzyme solution at a protein concentration of 0.12mg/ml were immersed briefly in solid CO_2/acetone mixtures before incubation in an ice bath for various periods of time. Samples for cold-lability studies were taken from the ice bath in duplicate at the appropriate time-interval, and placed in a water bath at 37°C for 2min to equilibrate before being assayed for enzyme activity in the usual way. Samples which were to be re-activated by warming were also removed from the ice bath at equivalent time-intervals and placed in the water bath at 37°C for 30min before being assayed for arylsulphatase activity. Samples of enzyme left at room temperature (20°C) served as untreated controls in both cases. Fig. 3 shows that the arylsulphatase B sample was inactivated by freezing and that the loss of activity caused by cold treatment could be restored nearly completely by rewarming at 37°C for 30min. Further investigation revealed that this reversible loss of activity persisted for up to 24h exposure to cold treatment. Samples stored longer in the frozen state progressively became irreversibly inactivated, the activity being no longer recoverable by rewarming.

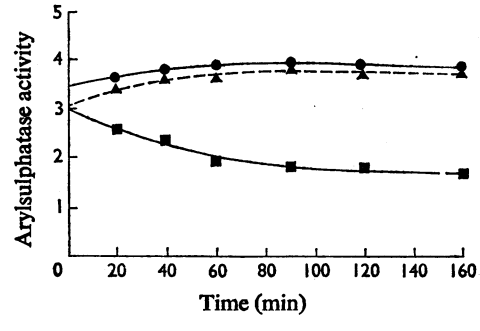

Fig. 3. *Kinetics of cold inactivation of purified arylsulphatase B*

Samples (0.5ml) of the enzyme solution (0.12mg of protein/ml) were incubated at 0°C for the various times indicated. After incubation, samples were either assayed directly for activity or were rewarmed at 37°C for 30min before assay, ●, Activity of control untreated enzyme; ▲, activity of enzyme after cold treatment and subsequent rewarming; ■, activity of cold-treated enzyme. Arylsulphatase activity is expressed in arbitrary units.

Preliminary analysis of the molecular-weight distribution of the protein by ultracentrifugation showed that whereas untreated enzyme still had a mol.wt. of 55000–60000, the cold-treated enzyme was polydisperse, showing values from 10000 to 46000 and also very-high-molecular-weight components.

Discussion

The non-availability of homogeneous preparations of arylsulphatase B has hampered to some extent the elucidation of the functional significance of the mammalian arylsulphatases. This situation is particularly critical for arylsulphatase B of human tissues, where recent reports of hydrolysis of UDP-*N*-acetylgalactosamine 4-sulphate by arylsulphatase B (Fluharty *et al.*, 1975) have relied on enzyme preparations partially purified by repeated ion-exchange chromatography (Allen & Roy, 1968).

The development of an affinity-chromatography procedure and its application to the purification of arylsulphatase B described in the present paper has led to the preparation of enzyme which appeared pure on electrophoresis and ultracentrifugation. The ligand 4-hydroxy-2-nitrophenyl sulphate has been shown to be specific for arylsulphatase B (Agogbua & Wynn, 1975) and the true affinity nature of the matrix can be confirmed by the fact that 2-nitroquinol sulphate may be replaced by a number of the usual substrates for the enzyme with retention of the effect.

Throughout this investigation only one form of arylsulphatase B has been detected. This is surprising in view of the reports of the existence of multiple forms of the enzyme in various mammalian tissues (Wortman, 1962; Bleszynski, 1967; Allen & Roy, 1968; Jerfy & Roy, 1973). In the stage of purification on CM 52 CM-cellulose a loss of approx. 60% of the applied activity was always observed. It may be that another form of arylsulphatase B is present, which is not eluted under our present conditions, although extensive washing at high ionic strength and pH failed to elute any further activity. The reason for the existence of such multiple forms is as yet unclear. In some preparations, the tissue has been allowed to autolyse and degradative changes brought about by the action of various enzymes such as proteinases and neuraminidase may have led to the presence of isoenzymes (see Goldstone & Koenig, 1972). Cellular and lysosomal heterogeneity can also lead to apparent multiple forms of the enzyme.

The kinetic data of the purified enzyme are similar to those reported previously (Dodgson & Wynn, 1958). The loss of activity on freezing has not been systematically investigated previously. The loss of activity on cold storage of pyruvate carboxylase was suggested to be due to dissociation and/or re-aggregation of the enzyme into inactive molecular forms (Irias *et al.*, 1969). Such an explanation would be consistent with the preliminary observations of changes in ultracentrifuge pattern of purified arylsulphatase B after cold treatment. The enzyme could dissociate into inactive promoters in the cold and this process could be reversed on warming. On prolonged standing at cold temperatures, aggregation of protomers into inactive polymers of varying size could occur. The residual activity observed would represent the proportion of protomers still available for aggregation to active enzyme.

The results of the effects of amino acid group-specific reagents and photo-oxidation, although not conclusive, point to the involvement of histidine residues in the catalytic activity of the enzyme. These results are in agreement with those obtained from the variation of K_m and V with pH (Dodgson & Wynn, 1958). Model studies by Benkovic (1971) and Kiefer *et al.* (1972) with synthetic polymers have shown the feasibility of the catalytic role of histidine residues in the active site of arylsulphatases. Jerfy & Roy (1969) in a similar study of the ox liver arylsulphatase A with group-specific reagents suggested the involvement of histidine residues at the active site possibly concerned with the maintenance of protein conformation compatible with enzyme activity. In contrast with the findings of Jerfy & Roy (1969), our enzyme was not inhibited by *N*-acetylimidazole and cannot be dependent on tyrosine residues at the active site.

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