The Sulphatase of Ox Liver

2. THE PURIFICATION AND PROPERTIES OF SULPHATASE A

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The first paper of this series (Roy, 1953) reported the presence of two distinct fractions exhibiting sulphatase activity in an aqueous extract of an acetone powder of ox liver, and gave preliminary data on the properties of the two crude enzymes, which were separated by fractional precipitation with acetone. The present paper describes the further purification and properties of fraction *A*, now referred to as sulphatase *A*.

EXPERIMENTAL

Preparation of sulphatase A

The starting material was an acetone powder of ox liver prepared as described previously (Roy, 1953). Cold acetone fractionation was carried out in an apparatus similar to that described by Askonas (1951) using thin aluminium vessels to ensure rapid heat transfer. Precipitation with $(\text{NH}_4)_2\text{SO}_4$ was effected by adding to the solution, with constant stirring, the calculated amount of solid $(\text{NH}_4)_2\text{SO}_4$; after the salt had dissolved the mixture was kept 4–6 hr. at room temperature before the precipitated protein was removed by centrifuging. Enzyme solutions were dialysed in Visking cellophan tubing against running tap water at room temperature unless otherwise specified.

Unfractionated extract. 60 g. of the acetone powder were incubated for 1 hr. at 37° with 400 ml. water and the insoluble material removed by centrifuging. The debris was washed with a further 150 ml. water and the combined supernatants were clarified by centrifuging for 30 min. at 8000 g.

Stage A-2. To 400 ml. unfractionated extract was added 40 ml. 0.2 M-phosphate buffer, pH 7.0 (final pH 6.8) and the volume made up to 450 ml. After bringing to 0°, 340 ml. cold acetone were slowly added, the temperature being lowered to –9° during the process. After equilibration at –9° for 30 min., the precipitate of fraction *B* (Roy, 1953) was centrifuged off at the same temperature and discarded. To the supernatant (650 ml.) kept at –9° were added 15 ml. of the phosphate buffer, followed by a further 280 ml. of acetone. The mixture was equilibrated as before and the precipitate of crude sulphatase *A* centrifuged off at –9°, dissolved in 75 ml. water and dialysed overnight to give approximately 140 ml. of a clear red solution of sulphatase *A*.

Stage A-1. To 140 ml. *A* were added 10 ml. 0.5 M-sodium acetate, pH 6.5 (final pH 7.0) and 1.5 ml. 0.3 M- CaCl_2 . The solution was then precipitated with 115 ml. acetone at –9° as described above, and the inactive precipitate discarded; the supernatant, kept at –9°, was treated with a further 115 ml. acetone, the precipitate centrifuged off as before,

Table 1. *The course of a typical preparation of sulphatase A*

Stage	Total activity (s.u.)	Activity (s.u./mg. N)
Unfractionated extract	126 000	38
<i>A</i>	150 000	330
<i>A-1</i>	120 000	3 000
<i>A-2</i>	90 000	—
<i>A-3</i>	40 000	6 700
<i>A-4</i>	29 000	40 000

dissolved in 25 ml. water, and dialysed overnight to give 40 ml. of a solution of sulphatase *A-1*.

Stage A-2. Sufficient solid $(\text{NH}_4)_2\text{SO}_4$ was added to *A-1* to make the solution 30% saturated $(\text{NH}_4)_2\text{SO}_4$. After standing 5 hr., the active precipitate was centrifuged off, dissolved in 10 ml. water and dialysed overnight.

Stage A-3. *A-2* was 20% saturated with $(\text{NH}_4)_2\text{SO}_4$, and the inactive precipitate centrifuged off. The supernatant was then 40% saturated with $(\text{NH}_4)_2\text{SO}_4$, the precipitate separated, dissolved in 5 ml. water and dialysed overnight, giving 8 ml. of a clear, faintly straw-coloured liquid.

Stage A-4. The clear solution of *A-3* was dialysed at 0° against twelve or more changes of distilled water for 48 hr. and the white, flocculent precipitate of the enzyme was centrifuged off. This was extracted for 24 hr. at 0° with 4 ml. 0.1 M- NaCl , during which time the bulk of the enzyme passed into solution. The insoluble residue was centrifuged off, washed with 0.1 M- NaCl , and the combined supernatants stored at –10°.

The concentrated enzyme solutions so obtained were perfectly stable, even at 0°, but when diluted to a concentration suitable for assay as described below, the enzyme rapidly lost its activity. When so diluted, about 50% inactivation occurred on standing 24 hr. at 0° in the case of sulphatase *A-4*. Less highly purified preparations were considerably more stable.

Table 1 shows the course of a typical preparation of sulphatase *A*. One sulphatase *A* unit (s.u.) is defined as the amount of enzyme which, under the standard conditions described below, liberates $(1 \mu\text{g.})^{\frac{1}{2}}$ nitrocatechol. The reason for raising the amount of nitrocatechol liberated to the power of $\frac{1}{2}$ is described below.

Preparation of sulphuric acid esters

The majority of the sulphuric acid esters were prepared by the method of Burkhardt & Lapworth (1926) using the chlorosulphonic acid-dimethylaniline complex in CHCl_3 solution as the sulphating agent. Dipotassium 2-hydroxy-5-nitrophenol sulphate (nitrocatechol sulphate) was prepared

as described previously (Roy, 1953). Steroid sulphates were prepared by sulphation with pyridine:sulphur trioxide (private communication from Dr J. Y. F. Paterson) as were the monosaccharide sulphates (Duff, 1949).

Estimation of enzymic activity

Routine estimations were carried out using the method previously described (Roy, 1953) with nitrocatechol sulphate as substrate. The total volume of the reaction mixture was 0.8 ml. containing 0.2 ml. enzyme solution, 0.2 ml. 0.5 M-acetate buffer, pH 4.9, and 0.4 ml. 0.006 M-nitrocatechol sulphate adjusted to pH 4.9 with 0.1 N-HCl, giving a final substrate concentration of 0.003 M-nitrocatechol sulphate in 0.13 M-acetate buffer. After incubating for 1 hr. at 37°, the solution was deproteinized with 3 ml. 2% phosphotungstic acid in 0.1 N-HCl and the liberated nitrocatechol estimated colorimetrically by means of the red colour developed in an alkaline quinol solution. Assays were run in duplicate along with appropriate enzyme and substrate blanks. During typical assays approximately 50 µg. nitrocatechol were liberated, corresponding to a 10% hydrolysis of the substrate. Under the above conditions the degree of hydrolysis was linearly related to the time of incubation for periods of at least 2 hr. in the case of enzyme solutions purified to stage A-3. Some preparations purified to stage A-4 gave a decreasing rate of hydrolysis after 1 hr. incubation.

Use of substrates other than nitrocatechol sulphate. In all cases the general technique of the enzyme assay was similar to that described above, the principal difference being in the methods of estimating the liberated phenols. In the case of simple phenolic compounds (phenol, cresols, naphthols, 2-phenanthrol) the phenol was estimated colorimetrically using Folin & Ciocalteu reagent as described by Kerr, Graham & Levy (1948) for the estimation of β -glucuronidase. To the reaction mixture at the end of incubation was added 3 ml. Folin & Ciocalteu reagent diluted 1 in 5 with water. Any precipitated protein was centrifuged off and 3 ml. of the supernatant pipetted into 5 ml. N-Na₂CO₃ for colour development. After standing for 15 min. at 37°, the blue colour was read in the Spekker absorptiometer using Ilford filter no. 608 (700 m μ). With *p*-nitrophenyl sulphate, the liberated *p*-nitrophenol was estimated colorimetrically by means of the yellow colour developed in alkaline solution. The assay was similar to that described for nitrocatechol sulphate except that 3 ml. of the supernatant from the phosphotungstic acid precipitation was pipetted into 5 ml. N-Na₂CO₃ for colour development. The yellow colour was read in the Spekker absorptiometer using Ilford filter no. 601 (425 m μ).

Study of inhibitory substances

The substance under consideration was dissolved in the 0.5 M-acetate buffer used in the routine assay described above to give any required concentration, after which the pH was checked (glass electrode) and if necessary adjusted to pH 4.9 with 0.1 N-HCl or NaOH. The buffered inhibitor solutions so obtained were used in place of the normal buffer in the routine assay, so that in all cases the enzyme was added to the previously mixed substrate and inhibitor.

RESULTS

In all the following experiments the enzyme used had been purified as far as stage A-3, unless otherwise specified in the text.

Effect of enzyme concentration on the reaction velocity. In the previous communication it was reported that in the case of crude sulphatase A (stage A) the rate of hydrolysis of nitrocatechol sulphate was not directly proportional to the enzyme concentration, whereas the relation was a linear one in the case of fraction B. It was at that time suggested that this anomaly might be due to the crude nature of the fraction, but it has now been found that highly purified preparations of sulphatase A also exhibit this effect, as is shown in Fig. 1. The effect is not due to the presence of an activator or coenzyme, as the addition of boiled enzyme solution to the assays makes no difference to the response to changes in enzyme concentration.

Fig. 1. Effect of enzyme concentration on reaction velocity. Final volume of reaction mixture: 0.8 ml., containing 0.2 ml. enzyme solution of varying concentration, 0.4 ml. 0.006 M-nitrocatechol sulphate and 0.2 ml. 0.5 M-acetate buffer, pH 4.9. Incubated 1 hr. at 37°.

Fig. 2 shows that although the reaction velocity is not proportional to the enzyme concentration it is, at least approximately, so related to the enzyme concentration raised to the power of $\frac{3}{2}$ over the range of enzyme concentration usually studied. This relationship appears to break down at higher enzyme concentrations, where the reaction velocity is even higher than would be expected from the relationship $v = kE^{\frac{3}{2}}$ (v is reaction velocity, E the enzyme concentration, and k a constant). Table 2 illustrates this point.

Fig. 2. Relationship between reaction velocity and the enzyme concentration raised to the power $3/2$ ($E^{3/2}$). Conditions as in Fig. 1. The figures on the lines indicate the state of purification of the enzyme.

Table 2. Effect of enzyme concentration on reaction velocity

E	$E^\ddagger$	$v_{\text{obs.}}$	$\frac{v}{E^\ddagger}$	$v_{\text{calc.}}^\dagger$
0.10	0.032	1.3	40.6	1.2
0.20	0.089	3.5	39.4	3.3
0.25	0.125	4.3	34.4	4.7
0.30	0.164	6.0	36.6	6.2
0.40	0.253	8.5	33.6	9.5
0.50	0.353	13.0	36.8	13.8
0.75	0.650	25.5	39.3	24.5
1.0	1.00	39.5	39.5	37.5
2.0	2.83	134.0	47.4*	106.0
3.0	5.20	243.0	46.8*	195.0
4.0	8.00	370.0	46.3*	300.0

* Omitted from calculation of mean value of $\frac{v}{E^\ddagger}$.

$\dagger v_{\text{calc.}} = kE^\ddagger$, where k is the mean value of $\frac{v}{E^\ddagger}$.

It follows from the above relationship that in the case of enzyme assays at normal enzyme concentrations (liberating some 50 $\mu\text{g.}$ nitrocatechol under the standard conditions) it is necessary to raise the amount of nitrocatechol liberated to the power of $\frac{3}{2}$ to obtain an approximate measure of the amount of enzyme present. One sulphatase unit is therefore defined as the amount of enzyme liberating (1 $\mu\text{g.}$) ‡ of nitrocatechol under the standard conditions described above.

Effect of changes in pH. The effect of varying pH on the activity of the enzyme is shown in Fig. 3, the assays being carried out in 0.13 M-acetate buffers at a final substrate concentration of 0.003 M-nitrocatechol sulphate. It is obvious from the figure that

sulphatase *A* has a well-defined optimum at pH 4.9 in acetate buffers. With different preparations of the enzyme, the position of the optimum varied between pH 4.9 and 5.0. Fig. 3 also shows the effect of citrate buffers on the pH optimum of sulphatase *A*. In 0.13 M-citrate the pH optimum is displaced to approximately pH 5.9, and the reaction velocity at lower pH values is considerably depressed, compared with the velocity in acetate buffers of corresponding pH values. It would appear that the optimum at pH 5.9 in citrate is only an apparent one brought about by the very strong inhibition of the enzyme by citrate below pH 6.

Fig. 3. Effect of pH on reaction velocity. Incubated for 1 hr. at 37° in 0.15 M-acetate or citrate buffers of varying pH. Substrate concentration 0.003 M-nitrocatechol sulphate. Final volume of reaction mixture 0.8 ml. containing 0.2 ml. enzyme solution. —●—, acetate buffer, —×— citrate buffer.

Although several early preparations of sulphatase *A* gave pH-activity curves of the above type, more recent preparations have given curves of a very different nature, typical examples of which are shown in Fig. 4. This change is not due to any detectable alteration in the experimental procedure, which has been kept constant throughout the investigations, and it has not so far proved possible to repeat the preparations of specimens of sulphatase *A* with a simple pH curve. The complexity of the problem is indicated in Fig. 4, which shows that the shape of the pH curve varies markedly with changes in enzyme concentration. At high enzyme concentrations the enzyme activity is greatest at pH 5.2–5.3 with a subsidiary optimum at pH 4.8–4.9: as the enzyme concentration is lowered this latter optimum becomes more prominent and the optimum at pH 5.2 becomes subsidiary. Finally, in low enzyme concentrations the optimum at pH 5.2 disappears, that at pH 4.8 becomes less prominent, and the major optimum appears at pH 4.5–4.6. That is, as the enzyme concentration is lowered, the optimum shifts from pH 5.2 through pH 4.8 to 4.5.

Fig. 4. Effect of varying enzyme concentration on the pH-activity curves. Conditions as in Fig. 3 except that the concentration of the enzyme was varied, and only acetate buffers were used. The figures on the curves indicate the relative enzyme concentrations.

This effect is quite constant, and ten entirely independent preparations at varying stages of purity have given similar results. Unfortunately, samples of the earlier preparations were not available for investigation of this problem.

The inhibition of sulphatase *A* by citrate suggested that the enzyme might require magnesium ions as an activator. As Smith (1950) showed that the electrophoretic behaviour of leucine aminopeptidase was altered in the presence of its activator, manganese, it seemed possible that the presence of magnesium might alter the shape of the pH-activity curve of sulphatase *A*, as any change in electrophoretic behaviour is likely to be accompanied by a corresponding shift in the position of the pH optimum. The effect of magnesium ions on the pH-activity curve of the enzyme was therefore investigated. No significant difference could be detected between the pH curve of the normal enzyme and enzyme which had been treated with 0.01 *M* magnesium chloride for 30 min. before assay.

Effect of changes in substrate concentration. Fig. 5 shows the effect of varying substrate concentration on the reaction velocity. As previously reported for crude fraction *A*, the reaction velocity reached a maximum at a substrate concentration of 0.003 *M* nitrocatechol sulphate. Fig. 5 also gives the results of a typical experiment plotted according to the method of Lineweaver & Burke (1934) and allows

Fig. 5. Effect of varying substrate concentration on the reaction velocity. Incubated for 1 hr. at 37° with varying concentrations of nitrocatechol sulphate in 0.15 *M* acetate buffer, pH 4.9. Final volume of reaction mixture 0.8 ml. containing 0.2 ml. enzyme solution. ●—●, plot of reaction velocity against substrate concentration; x—x, the above data plotted according to the equation derived by Lineweaver & Burke (1934)

$$\frac{s}{v} = \frac{1}{V} s + \frac{K_m}{V},$$

where *s* is the substrate concentration, *v* and *V* the observed and maximum velocities respectively, and *K_m* the Michaelis constant.

Fig. 6. Inhibition by Na_2SO_4 and Na_2SO_3 . Conditions as in Fig. 5. Inhibitor concentration as indicated on the figure. Plotted according to the equation derived by Hunter & Downs (1945)

$$I \frac{\alpha}{1-\alpha} = K_i + \frac{K_i}{K_m} s,$$

where *I* and *s* are the inhibitor and substrate concentrations, α the fractional activity, *K_m* the Michaelis constant, and *K_i* the dissociation constant of the enzyme-inhibitor complex.

the calculation of the Michaelis constant, K_m . In the experiment shown the value of K_m is 7.9×10^{-4} M-nitrocatechol sulphate; a further two experiments gave values of 8.2×10^{-4} M- and 7.6×10^{-4} M-nitrocatechol sulphate.

As the above determinations were carried out with one of the early preparations, the experiments were repeated using a more recent enzyme preparation having multiple pH optima. The effect of substrate concentration was investigated with high and low concentrations of enzyme, at pH 5.2 and 4.6 respectively. As in both cases the results were very similar to those shown in Fig. 5 they are not reported here in detail. The optimum substrate concentration was 0.003 M-nitrocatechol sulphate with both enzyme concentrations, but the values of K_m appeared to be significantly higher than those reported above, being 12×10^{-4} M-nitrocatechol sulphate in both cases.

Effect of inhibitory substances. The following studies were carried out with an early enzyme preparation having a pH optimum of 5.0. Some of the results of the study of a large number of substances as possible inhibitors of sulphatase A are shown in Tables 3 and 4. Table 3 shows the effect of a number of compounds on the enzymic activity. In confirmation of the results of Tanaka (1938) for molluscan phenol sulphatase, sodium sulphate was found to be a powerful inhibitor of sulphatase A, which therefore differs in this respect from the phenol sulphatase of Taka diastase (Taka sulphatase) as the latter is not inhibited by sodium sulphate (Robinson, Smith, Spencer & Williams, 1952). Like Taka sulphatase, however, sulphatase A is strongly inhibited by sodium sulphite. Fig. 6 shows that the inhibition by both these substances is competitive and allows the calculation of the respective values of K_i by the method of Hunter & Downs (1945). These values are 7.5×10^{-4} M-sodium sulphate and 2.0×10^{-6} M-sodium sulphite respectively.

The other results listed in Table 3 show little of interest, except that 0.05 M-potassium cyanide does not inhibit sulphatase A, whereas Taka sulphatase is completely inhibited by this concentration of cyanide (Robinson *et al.* 1952). Like Taka sulphatase, sulphatase A is strongly inhibited by phosphate. Again, the activity of sulphatase A is uninfluenced by sodium chloride, thus distinguishing it from the phenol sulphatase of the limpet (*Patella vulgata*) which is activated by this substance (Dodgson & Spencer, 1952). The activation of rat-liver sulphatase by potassium chloride reported by Dodgson, Spencer & Thomas (1953) is probably not a true activation, but rather an apparent one brought about by a change in the state of dispersion of the enzyme studied by these authors. Potassium chloride is without effect on sulphatase A.

Table 3. *Effect of various compounds on the activity of sulphatase A*

(Final volume of reaction mixture 0.8 ml. containing 0.2 ml. 0.5 M-acetate buffer, pH 4.9, 0.4 ml. 0.006 M-nitrocatechol sulphate and 0.2 ml. enzyme solution. The inhibitor was dissolved in the acetate buffer to give the required concentration.)

Compound	Final concentration (M)	Inhibition (%)
Na_2SO_4	0.025	85
	0.0025	40
	0.25×10^{-3}	100
Na_2SO_3	0.25×10^{-3}	90
	0.0025×10^{-3}	5
	0.05	0
NaCl	0.05	0
KCl	0.05	0
MgCl_2	0.1	15
	0.01	0
BaCl_2	0.025	40
KCN	0.01	2
NaF	0.01	95
KH_2PO_4	0.025	100
$\text{C}_6\text{H}_5\text{PO}_4\text{Na}_2$	0.025	80
$\text{C}_6\text{H}_5\text{SO}_3\text{K}$	0.05	10
$\text{C}_6\text{H}_5\text{SO}_3\text{K}$	0.05	0

Table 4. *Effect of various sulphuric acid esters on the activity of sulphatase A*

(Experimental conditions as in Table 3.)

Compound	Concentration (M)	Inhibition (%)	K_i
Potassium methyl sulphate	0.05	0	—
Potassium benzyl sulphate	0.05	10	—
Potassium cyclohexyl sulphate	0.05	3	—
Potassium phenyl sulphate	0.05	10	3×10^{-1}
Potassium <i>m</i> -tolyl sulphate	0.05	30	1×10^{-1}
Potassium 1-naphthyl sulphate	0.01	18	4×10^{-2}
Potassium 2-naphthyl sulphate	0.01	30	3×10^{-2}
Potassium 2-phenanthryl sulphate	0.002	30	4×10^{-3}
Potassium androsterone sulphate	0.005	30	1×10^{-2}
Sodium glucose 6-sulphate	0.025	0	—
Sodium glucose 3-sulphate	0.025	0	—
Sodium chondroitin sulphate	10 mg./ml.	0	—
Heparin	10 mg./ml.	0	—

Table 4 shows the effect of several sulphuric acid esters on the activity of sulphatase *A*. It is immediately obvious that only the esters of the more complex compounds exert any significant inhibitory action, there being a definite decrease in the value of K_i and therefore an increase in the affinity of the enzyme for the inhibitor with an increase in complexity towards a polycyclic alkyl or aryl sulphate. None of the carbohydrate sulphates so far studied exhibited any inhibitory effect on the enzyme.

Fig. 7. Effect of pH on the hydrolysis of *m*-tolyl sulphate. General conditions as in Fig. 3 except that the substrate concentration was 0.1 M *m*-tolyl sulphate.

Fig. 8. Plot of log reaction rate ($\log v$) against reciprocal of absolute temperature ($1/T$) for varying concentrations of sulphatase *A*. Final volume of reaction mixture 0.8 ml. containing: 0.2 ml. enzyme solution, 0.2 ml. acetate buffer, pH 5.2 and 4.6 in the high and low enzyme concentrations respectively; and 0.4 ml. 0.006 M-nitrocatechol sulphate. Incubated for 1 hr. ●—●, relative enzyme concentration 6; —×, relative enzyme concentration 1.

The degree of inhibition is independent of the concentration of the substrate for any given inhibitor concentration, at least over the relatively small range of substrate concentration (0.00075 M to 0.003 M-nitrocatechol sulphate) so far studied. This may be taken as an indication that the inhibition by these sulphuric acid esters is non-competitive. This inhibition cannot, however, be completely non-competitive because, as described below, at least some of these aryl sulphates are slowly hydrolysed by the enzyme.

Hydrolysis of aryl sulphates. This problem has not yet been studied exhaustively, but the results indicate that sulphatase *A* hydrolyses the simpler aryl sulphates so far studied at a much lower rate than it does nitrocatechol sulphate. Also the affinity of the enzyme for these sulphates is low: in the case of *p*-nitrophenyl sulphate and *m*-tolyl sulphate the values of K_m were 0.04 and 0.2 M respectively, compared with a value of 0.0008 M for nitrocatechol sulphate. Again, the optimum pH for the hydrolysis of these sulphates is at approximately pH 5.7 in 0.13 M-acetate buffer, compared with the optimum at pH 4.9 for the hydrolysis of nitrocatechol sulphate. The pH-activity curve for the hydrolysis of *m*-tolyl sulphate is shown in Fig. 7.

As with nitrocatechol sulphate, early enzyme preparations gave a simple pH curve for the hydrolysis of the above sulphates, while more recent preparations have given multiple optima, the major optimum, however, being at pH 5.7. Relative rates of hydrolysis have not been accurately determined, but it appears that both *m*-tolyl and *p*-nitrophenyl sulphates are hydrolysed from 10 to 20 times more slowly than nitrocatechol sulphate by sulphatase *A*. Pilot experiments have indicated that the rates of hydrolysis of 1- and 2-naphthyl sulphates and 2-phenanthryl sulphate are of the same order as that for the hydrolysis of *m*-tolyl sulphate.

Energy of activation of sulphatase *A*. The energy of activation was determined at several enzyme concentrations, in each case the enzyme activity being assayed at the optimum pH for the appropriate enzyme concentration. Assays were carried out in 0.003 M-nitrocatechol sulphate at various temperatures between 15 and 37°. Fig. 8 shows the results of a typical experiment plotted according to the Arrhenius equation (Arrhenius, 1889)

$$\ln \frac{k_2}{k_1} = \frac{A}{R} \left(\frac{1}{T_1} - \frac{1}{T_2} \right),$$

where k is the reaction rate at absolute temperature T , R is the gas constant and A the energy of activation. From the data of Fig. 8 the energy of activation may be calculated, and in the experiment shown the values were 6500 cal./mole and 14 000 cal./mole

at high and low enzyme concentrations respectively (relative enzyme concentrations 6 and 1, corresponding to the upper and lower curves of Fig. 4).

DISCUSSION

The reported method of purification gives a very considerable concentration of the enzyme, as is indicated in Table 1; the final yield at stage A-4 is approximately 20% of the enzyme present at the stage of the unfractionated extract and represents 1000-fold concentration of the enzyme with respect to protein nitrogen. Even at stage A-4, however, the enzyme is not pure, as the electrophoretic pattern of a typical preparation determined at pH 6.5 in the Hilger Tiselius apparatus indicates the presence of at least four components. In order to determine which of the components represented sulphatase A, electrophoresis was carried out on filter-paper strips, using an apparatus essentially similar to that described by Latner (1952). The bulk of the enzymic activity did not appear to be associated with the major component. It may therefore be concluded that, although a considerable degree of purification has been attained, stage A-4 does not represent a pure enzyme.

It should be noted that the method of preparation shows that sulphatase A is a globulin, as it is almost quantitatively precipitated by 30% saturated ammonium sulphate and is insoluble in distilled water. Sulphatase A therefore differs markedly from Taka sulphatase which Dzialoszynski (1951) has shown to be an albumin.

The anomalous relationship between enzyme concentration and reaction velocity, shown in Fig. 1 and Table 3, is very unusual. It is difficult to explain this effect but the following mechanism seems a probable one, the more so as it has a bearing on the multiple nature of the pH curve. The basic postulates are two in number:

(1) That the enzyme molecules (E) are capable of associating reversibly with one another to form a dimeric enzyme (E_2)

The dissociation constant of this reaction is given by

$$K = \frac{n_2}{n_1^2} \quad (1)$$

where n_1 and n_2 are the concentrations of E and E_2 respectively.

(2) That the dimeric form of the enzyme is a more efficient catalyst than is the monomeric form, i.e. $k_2 > k_1$ in the following reactions:

If the above postulates are valid, then the velocity of the enzyme reaction is given by

$$\begin{aligned} -\frac{dn_s}{dt} &= (k_1 n_1 + k_2 n_2) n_s \\ &= (k_1 n_1 + k_2 K n_1^2) n_s \end{aligned} \quad (2)$$

where n_s is the concentration of the substrate. Or, in terms of a total enzyme concentration, n , reckoned as monomeric units, and α , the fraction of the total amount of enzyme which is present in the dimeric form

$$n_1 = (1 - \alpha) n, \quad n_2 = \frac{1}{2} \alpha n.$$

Substituting the above in (2)

$$-\frac{dn_s}{dt} = [k_1(1 - \alpha) n + k_2 K(1 - \alpha)^2 n^2] n_s \quad (3)$$

where the value of α is given by the expression

$$\alpha = 1 + \frac{1}{4Kn} - \sqrt{\left[\left(1 + \frac{1}{4Kn} \right)^2 - 1 \right]} \quad (4)$$

obtained by substituting the above expressions for n_1 and n_2 in terms of n and α into equation (1) and solving the quadratic so obtained for α .

It is obvious from equations (3) and (4) that under such conditions the reaction velocity would be proportional not to the enzyme concentration, but to some power, intermediate between the first and second, of the enzyme concentration. Further, if the enzyme were capable of polymerizing in several stages — E , E_2 , E_3 , ..., E_n — the relationship shown in equation (3) would be of the same general form, provided that the second of the above postulates remained valid.

It must be stressed that direct evidence for the above theory will be difficult to obtain until the enzyme is obtained in a pure state, and the above explanation must be regarded as tentative, the more so as no explanation can be offered as to why the original preparations gave a simple pH curve with a single optimum at pH 5, while more recent preparations have shown multiple pH optima.

The first of the above postulates, however, is by no means unlikely as many examples of protein-protein interactions have now been described. The most studied example is that of insulin which has definitely been shown (Gutfreund, 1948, 1952) to exist in various polymerized forms of a simple subunit; likewise Pedersen (1950) has described similar phenomena in bovine CO-haemoglobin.

The second postulate is at first sight less possible, as polymerization of the simple molecules must decrease the absolute number of enzymically active particles present, which must in turn decrease the chance of combination between the enzyme and its substrate, and so might be expected to reduce the enzymic activity, rather than to increase it. That this is apparently not the case is indicated by the

results shown in Fig. 8 and the values of the activation energies calculated therefrom. At high enzyme concentrations the activation energy is around 7000 cal./mole whereas at low concentrations the activation energy rises to 14 000 cal./mole. This implies that at high concentrations the enzyme is more catalytically active than at low concentrations, and so supports the second of the above two postulates. It must be stressed that the above values of the activation energy have little absolute significance as intermediate values may be obtained, presumably due to the fact that it is impossible to obtain an enzyme solution completely homogeneous with respect to any given polymeric form and therefore the values refer to solutions containing two or more molecular species of enzyme.

Further evidence for the probable truth of the above hypothesis is given by a consideration of the pH-activity curves shown in Fig. 4 which indicate a definite shift in the pH optimum with changes in enzyme concentration. If the enzyme can exist in a monomeric form and one or more polymerized forms then it is not unlikely that each of the forms would have a definite pH optimum. From the results shown in Fig. 4 it seems justifiable to conclude that the pH optimum of the simple, unpolymerized form is approximately pH 4.6 and with increasing degrees of polymerization of the enzyme the pH optimum moves to pH 4.9 and then to pH 5.2.

From the inhibitory action of citrate and fluoride on the enzyme it seems probable that sulphatase *A* requires magnesium ions for its complete activity. This conclusion would not be expected from the findings of Hommerberg (1931) and of Tanaka (1938), both of whom claimed that magnesium chloride inhibited sulphatases from a number of animal tissues: this inhibition is, however, confirmed by the results shown in Table 3 which indicate that 0.1M-magnesium chloride inhibits sulphatase *A* slightly. Lower concentrations were without effect, although some preparations were activated to a slight extent (not more than 10%) by 0.005M-magnesium chloride. This effect was unfortunately variable. On the other hand, dialysis of

stage *A-3* enzyme for 48 hr. against several changes of glass-distilled water did not significantly depress the enzymic activity, although the enzyme was precipitated during the dialysis. In this connexion, Seligman, Chauncey & Nachlas (1951) claimed that rat-liver sulphatase was activated by magnesium chloride: it seems, however, that these claims are of doubtful validity. The enzyme used by these workers was a 'homogenate' of formalin-fixed tissues, and the so-called activation was obtained by steeping the blocks of tissue in a solution of magnesium chloride before grinding: it seems probable that the effect can be explained by the steeping in the aqueous solution removing traces of formalin. This latter view is supported by the fact that Seligman *et al.* (1951) also claimed that $\text{Fe}_3(\text{SO}_4)_2 \cdot (\text{NH}_4)_2\text{SO}_4$ was an activator of the enzyme.

Only preliminary studies of the specificity of sulphatase *A* have as yet been carried out, but it is obvious from the data of Table 4 that the enzyme has very little affinity for the simpler aryl sulphates, and apparently none for the few carbohydrate sulphates so far studied. Also, the simpler aryl sulphates are hydrolysed much more slowly than is nitrocatechol sulphate. The considerable increase in the affinity of the enzyme for sulphates containing a polycyclic ring system is of interest as it suggests a possible role for the enzyme in steroid metabolism. Unfortunately, it has not been possible to investigate the action of the enzyme on oestrone sulphate, as no pure specimens of this substance were available, all samples being badly contaminated with inorganic sulphate, nor was it possible to synthesize oestrone sulphate in a sufficient state of purity by the methods of Butenandt & Hofstetter (1939) or Grant & Glen (1949). In this connexion, it is of interest that oestrone sulphate administered to rats is apparently very rapidly hydrolysed to oestrone (Hanahan & Everett, 1950), whereas similarly administered phenyl sulphate is excreted unchanged (Garton & Williams, 1949). Although sulphatase *A* is strongly inhibited by androsterone sulphate, and has a high affinity for that substance (Table 4) no hydrolysis of androsterone sulphate could be detected, even after 18 hr. incubation with a con-

Table 5. *Comparison of Taka sulphatase and sulphatase A*

(In both cases the substrate used was nitrocatechol sulphate.)

	Sulphatase <i>A</i>	Taka sulphatase
Type of protein	Globulin	Albumin*
pH optimum (acetate)	4.9	5.9
Optimum substrate concn.	0.003M	0.003M
K_m	$8 \times 10^{-4}\text{M}$	$3.5 \times 10^{-4}\text{M}$
Effect of 0.02M-KCN	No effect	100% Inhibition
Effect of 0.02M- Na_2SO_4	85% Inhibition	No effect
Relative rate of hydrolysis of <i>p</i> -nitrophenol sulphate	1/20	2

* Dzialoszynski (1951).

centrated enzyme solution. This is in accordance with the view (Fromageot, 1938) that the sulphatases so far described are aryl sulphatases, specifically hydrolysing phenolic sulphates and having no action on alcoholic sulphates, and contrasts sharply with the recent claim of Henry, Thevenet & Jarrige (1952) that a sulphatase in the gut of *Helix pomatia* readily hydrolyses dehydroepiandrosterone sulphate.

The above results stress the great differences between sulphatase *A* and any previously described sulphatase, especially Taka sulphatase. Table 5 summarizes the principal differences between sulphatase *A* and Taka sulphatase, the data for the latter being those of Robinson *et al.* (1952).

SUMMARY

1. A method is described for the purification of ox-liver sulphatase *A*. A 1000-fold purification is achieved.

2. Differences between the original and recent enzyme preparations are described. The original preparations had a pH optimum of 4.9–5.0 in 0.13 M-acetate buffer; more recent preparations have shown multiple optima at pH 4.6, 4.9 and 5.2.

3. In the recent preparations the pH optimum varies with the enzyme concentration. In high enzyme concentrations the major optimum is at pH 5.2 and in low enzyme concentrations at pH 4.6.

4. The enzymic activity is not directly proportional to the enzyme concentration, but to the

enzyme concentration raised to some power intermediate between the first and second (approximately the power $\frac{2}{3}$). It is suggested that this effect can be explained by polymerization of the enzyme molecules to give complexes which are more active enzymically than are the unpolymerized molecules.

5. The variation in pH optimum with enzyme concentration can also be explained on the above basis.

6. The optimum substrate concentration is 0.003 M-nitrocatechol sulphate and the K_m is 8×10^{-4} M-nitrocatechol sulphate.

7. Sulphatase *A* is competitively inhibited by sodium sulphate and sodium sulphite ($K_i = 7 \times 10^{-4}$ M and 2×10^{-6} M respectively).

8. Sulphatase *A* hydrolyses the simpler aryl sulphates at a much lower rate than it does nitrocatechol sulphate.

9. Sulphatase *A* is distinguished from the aryl sulphatase of Taka diastase.

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