The Activation of an Arylsulphatase from Ox Liver by Chloride and other Anions

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A few years ago an attempt was made by the senior author to purify the enzyme in ox liver which hydrolyses *p*-nitrophenyl sulphate. Considerable difficulty was experienced at first in obtaining consistent and intelligible activity figures during fractionation. The anomalies were found to be due to two factors: the enzyme was strongly inhibited by inorganic phosphate, so that the activity was appreciably reduced even by the amount of phosphate present in an aqueous extract of acetone-dried ox-liver powder; and the enzyme showed very little activity in the absence of chloride, so that it appeared to be lost on dialysis against water. Reasonable yields were obtained if activity determinations were carried out on samples dialysed against sodium chloride solution.

Since the work commenced, other purifications of arylsulphatase from ox liver have been reported, especially that of Roy (1953*b*) (arylsulphatase A) and Roy (1954) (arylsulphatase B). The latter enzyme has also been purified from human liver by Dodgson & Wynn (1958). However, the activation by chloride seemed of some interest and a detailed study of the effect of anions was undertaken; the results are reported in this paper.

In a number of cases it has been reported that the activity of arylsulphatases is affected by chloride. Dodgson, Spencer & Thomas (1953) stated that the particulate enzyme obtained in the course of fractionation studies with rat-liver homogenates was activated by potassium chloride, but later Roy (1953*b*) attributed this to 'a change in the state of dispersion of the enzyme studied by these authors'. Roy (1955) published pH curves obtained in the presence and in the absence of 0.08 M-chloride for both arylsulphatase A and B from ox liver. With arylsulphatase A the main effect is an inhibition by chloride of up to 10% at pH values above the optimum, which is shifted towards the acid side. The optimum pH for arylsulphatase B is also shifted by chloride, from pH 6.0 to 5.7; there is little change of velocity at the optimum pH, but there is about 30% activation by chloride at pH 5.8. The substrate used in Roy's experiments was nitrocatechol sulphate. Contrary to these findings for ox liver, Dodgson &

Wynn (1958) found that arylsulphatase B from human liver was considerably inhibited by chloride. Sodium chloride (0.01 M) produces 10% increase in activity of the arylsulphatase prepared from limpet viscera by Dodgson & Spencer (1952).

The stimulation of nitrophenylsulphatase activity by chloride and other ions reported in the present paper is a much larger effect than any hitherto reported for arylsulphatases.

MATERIALS AND METHODS

Preparation of enzyme

Fresh ox liver was acetone-dried; 5 lb. batches were usually used. The powder was finally dried over conc. H_2SO_4 and NaOH; the activity of the powder was stable over a period of months. A portion (600 g.) of powder was extracted with 10 l. of 0.1 M-acetate buffer, pH 5.1, with mechanical stirring for 2 hr.; after centrifuging the residue was stirred up with a further 2.5 l. of water and spun again. The combined dark-red extracts were brought to pH 6.0 and solid $(\text{NH}_4)_2\text{SO}_4$ was added to 45% saturation. After standing overnight at 4° the precipitate was centrifuged off and the supernatant brought to 70% saturation with solid $(\text{NH}_4)_2\text{SO}_4$. After standing again for 12 hr. at 4° the precipitate was centrifuged down in a refrigerated centrifuge, and it was dissolved in the minimum amount of water and dialysed against two changes of distilled water, one of 0.1 M-NaCl and a further one of distilled water. The solution was adjusted to pH 4.0 with N-HCl, heated to 38° over a period of 10 min. and held at 38° for a further 30 min. During this period a small precipitate formed. The preparation was brought to pH 5.0 with N-NaOH and the heavy dark-coloured precipitate spun off at 0° and rejected; the supernatant was now adjusted to pH 6.0 with N-NaOH (all pH values were measured with the glass electrode). To this was added 0.5 vol. of calcium phosphate gel (dry wt. 30 mg./ml.), which was spun off at 0° after 5 min. and rejected. The supernatant was brought to 70% saturation with solid $(\text{NH}_4)_2\text{SO}_4$, and allowed to stand overnight at 4° and centrifuged. The precipitate was suspended in water and dialysed successively against water, 0.01 M-acetate at pH 6.0, water, 0.01 M-acetate at pH 6.0 and two changes of glass-distilled water. A small precipitate was spun off, giving 300–400 ml. of clear straw-coloured enzyme. This stock enzyme, which was normally diluted five times for use in the kinetic studies described in this paper, was kept frozen at -20° and was completely stable under these conditions for at least 6 months.

Substrates, activators and inhibitors

Monopotassium *p*-nitrophenyl sulphate (NPS) was supplied by Sigma Chemical Co., St Louis, Mo., U.S.A. Estimation by acid hydrolysis of three different samples used in this work gave values (after correcting for free *p*-nitrophenol) of 84, 98 and 103% of the theoretical for $\text{NO}_2\cdot\text{C}_6\text{H}_4\cdot\text{OSO}_3\text{K}$. The free nitrophenol in these batches corresponded to 0.22, 2.7 and 0.1% hydrolysis respectively. Stock solutions of NPS for the enzymic experiments were adjusted to pH 5.6 with acetic acid or NaOH.

Dipotassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate, NCS) was prepared by Mr B. Slater by the method of Roy (1953*a*). It contained 0.5% of free nitrocatechol and gave 94% of the theoretical amount of nitrocatechol on acid hydrolysis, assuming the preparation to be the dihydrate. Such preparations have been shown to contain appreciable amounts of nitropyrogallyl disulphate (Dodgson & Spencer, 1956; Roy & Kerr, 1956).

NaCl, KCl, KI, KNO_3 , KSCN, KCN, Na_2SO_4 and citric acid, as well as the components of phosphate and acetate buffers, were AnalaR reagents. NaBr, NaF and KClO_3 were normal laboratory reagents supplied by British Drug Houses Ltd. or Hopkin and Williams Ltd.

Measurement of enzymic activity

Hydrolysis of NPS. In following the purification of the enzyme, and during some preliminary experiments, activity was measured by a method based on that of Huggins & Smith (1947). A portion (1 ml.) of *m*-acetate buffer (pH 5.1 unless otherwise stated), 1 ml. of *m*-NaCl and a suitable amount of enzyme were made up to 4 ml. and equilibrated at 38°. The reaction was started with 1 ml. of 6 mM-NPS and, after 1 hr., 5 ml. of *n*-NaOH was added; the *p*-nitrophenol was estimated in the Beckman Spectrophotometer at 400 μ .

A method based on the amount of product formed in a fixed time is unsatisfactory for kinetic studies, especially if rather long times of incubation are necessary. For all of the activation and inhibition studies described in this paper, the Beckman Ratio Recording Spectrophotometer DK-2 (Beckman Instruments Inc., Fullerton, Calif., U.S.A.) was used.

Fig. 1 shows the absorption spectrum of NPS and the corresponding free phenol in acetate buffer, pH 5.6. The peak in the near-ultraviolet is shifted from 278 μ in the sulphate to 318 μ in the free phenol. The biggest increase in absorption at pH 5.6 on hydrolysis of NPS is shown at

Fig. 1. Absorption spectrum of NPS and free *p*-nitrophenol. Spectra of 0.06 mM solutions in 0.2M-acetate, pH 5.6, measured at 38° in 1 cm. cells in the Beckman Ratio Recording Spectrophotometer.

about 330 μ ; it was decided to use the change in extinction at 330 μ as a measure of arylsulphatase activity. Because of the large absorption by substrate at this wavelength, all measurements were made against corresponding control solutions of NPS and the 20-fold photomultiplier was employed.

Reactions were carried out in a total volume of 3 ml. in 1 cm. quartz cells in the Beckman 92527 Temperature Regulated Cell Holder at 38°. The standard reaction mixture contained 0.5 ml. of *m*-acetate buffer, 0.5 ml. of *m*-NaCl, enzyme and 1 ml. of 6 mM-NPS (added last to start the reaction). The control cell contained a similar mixture except that the enzyme was replaced by water. Typical progress curves are shown in Fig. 2. There was always a rapid apparent increase in extinction for 60–90 sec.; this does not correspond to stage I of the anomalous arylsulphatase A kinetics studied by Baum, Dodgson & Spencer (1958), but is an artifact, since a similar progressive rise for a period of 2–3 min. is shown when *p*-nitrophenol is pipetted into NPS in acetate buffer. After this initial period the increase in extinction was linear for at least 15 min.; the slope of the curve between 2 and 8 min. was used as a measure of the rate of enzymic hydrolysis. Separate calibration constants were needed at each pH value and at each substrate concentration, since the absorption by nitrophenol at 330 μ is somewhat reduced in the presence of high concentrations of NPS. Calibration curves were prepared from mixtures of standard *p*-nitrophenol and NPS in acetate buffer; in each case Beer's Law was obeyed over the range of concentrations used. At pH 5.6 in 2 mM-NPS a change of extinction of 1 at 330 μ corresponded to the production, in 3 ml. incubation mixtures, of 67.5 μ g. of *p*-nitrophenol. Enzymic hydrolysis

Fig. 2. Progress curves for enzymic hydrolysis of NPS. A tracing of the record of an individual experiment with the Beckman Ratio Recording Spectrophotometer. Reaction mixtures (3 ml.) contained 0.17M-acetate buffer, pH 5.1, 0.17M-NaCl, mM-NPS and the following amounts of stock enzyme diluted five times: (1) 0.2 ml.; (2) 0.3 ml.; (3) 0.5 ml.; (4) 1.0 ml.; (5) 1.5 ml. The time base of the machine was driven by a 60-cycle synchronous motor by 50-cycle current and the divisions on the time scale represent intervals of 144 sec., except for curve 1, for which they represent 72 sec.

rates were proportional to enzyme concentration at least up to amounts of enzyme giving rates of 160 $\mu\text{g.}$ of *p*-nitrophenol/hr. With this standard test the activity of various batches of the stock solution of enzyme described above ranged from 600 to 800 $\mu\text{g.}$ of nitrophenol/hr./ml. of enzyme.

Hydrolysis of NCS. It has not proved possible to devise a continuous-recording procedure for following NCS hydrolysis, and a method based on that of Roy (1953a) was used. The enzymic reactions were carried out in a total volume of 3 ml. containing 0.5 ml. of *m*-acetate, pH 5.6, enzyme and 0.5 ml. of 0.05M-NCS (added last to start the reaction), at 38°. Incubation periods of either 10 or 15 min. were normally used, and the reaction was stopped by adding 5 ml. of fresh alkaline quinol reagent, prepared as described by Roy. Deproteinization was unnecessary with the purified enzyme and the phosphotungstic acid was omitted. The liberated nitrocatechol was determined spectrophotometrically at 520 $m\mu$, the extinction being measured against corresponding controls in which the enzyme was added after the alkaline quinol. Fig. 3 shows that hydrolysis rates as measured by this procedure were proportional to enzyme concentration at least up to 0.2 ml. of the stock enzyme. The specific activities of various batches towards NCS at pH 5.6 in the absence of chloride ranged from 5000 to 6600 $\mu\text{g.}$ of nitrocatechol/hr./ml. of stock enzyme.

Sulphate liberation. For most of the work hydrolysis was followed by the liberation of free phenol, but for the 'mixed-substrate' experiment sulphate production was followed by the method of Dodgson & Spencer (1953). The coloured solution obtained according to this procedure after the final centrifuging was diluted fourfold and the extinction at 500 $m\mu$ measured in the Beckman Spectrophotometer. A calibration curve was constructed by using measured amounts of standard K_2SO_4 solution containing up to 80 $\mu\text{g.}$ of SO_4^{2-} .

RESULTS

Activation by anions

Activation by chloride of the enzymic hydrolysis of NPS. Fig. 4 shows the dependence of the activity

Fig. 3. Effect of enzyme concentration on NCS hydrolysis. Procedure was as described in the text; reaction mixtures contained 0.17M-acetate, pH 5.6, 0.17M-NaCl and 8.3 mM-NCS, with the amounts of enzyme shown. Incubation was at 38° for 10 min.

of stock enzyme preparation on added NaCl at pH 5.1. All the early experiments were carried out at this pH, which is close to the optimum of 4.9 in acetate buffer reported by Roy (1953b) for arylsulphatase A. At this pH maximum activity is shown at about 0.2M-NaCl, the activity falling off slightly at higher concentrations. Half the maximum velocity is obtained at about 0.02M-NaCl. For the standard enzyme assay 0.17M-NaCl was chosen.

Specificity of activating anions. A number of univalent anions were found to be effective as activators. The activation curves for these ions at pH 5.6 are shown in Fig. 5. Of other univalent

Fig. 4. Effect of chloride concentration on enzymic hydrolysis of NPS at pH 5.1. Reaction mixtures (3 ml.) contained 0.17M-acetate buffer, mM-NPS and 0.2 ml. of stock enzyme. Rates of hydrolysis were measured in the Recording Spectrophotometer at 38° and are expressed relative to that in 0.17M-NaCl.

Fig. 5. Effect of a number of salts on enzymic hydrolysis of NPS at pH 5.6. Reaction mixtures (3 ml.) contained 0.17M-acetate buffer, 2 mM-NPS and 0.2 ml. of stock enzyme. Rates of hydrolysis at 38° are expressed relative to that in a control containing 0.17M-NaCl. ■, No added salt (mean of nine experiments); ○, NaCl; ●, KCl; △, NaBr; ▲, KI; □, KSCN; ×, KClO_3 ; +, KNO_3 .

ions investigated, cyanide has no effect and fluoride is a powerful inhibitor (see below). Most of the activation experiments were carried out in 0.17M-acetate buffer and the question arises whether acetate ion has any influence on the activity. In an experiment with enzyme and substrate carefully adjusted to pH 5.6, the same rates were obtained in the absence of buffer and in 33 mM-, 67 mM-, 0.17M- and 0.33M-acetate; thus there was no evidence that acetate ion was capable of activating the enzyme. No evidence was seen that acetate had any effect on the activation by chloride.

The enzyme was unaffected by Ca^{2+} or Mg^{2+} ions, and the nature of the alkali-metal ion present appeared to have no effect. Fig. 5 shows that the same activity was obtained in the presence of KCl or NaCl.

Interpretation of Fig. 5 in terms of affinity constants is complicated by the inhibition shown by higher concentrations of some of these salts, especially thiocyanate, nitrate and, to a lesser extent, iodide. At concentrations lower than those at which this inhibition becomes noticeable, the curves are more or less rectangular hyperbolas. It was found that by plotting the reciprocal of the activity (corrected for the small activity without added salt) against the reciprocal of the concentration of salt added, straight-line portions could be obtained; the reciprocals of the intercepts obtained by extrapolating these straight-line portions are shown in Table 1. The extrapolated maximum velocities are probably not significantly different, with the exception of that for chlorate; unfortunately this ion was not tested at higher concentrations because of the limited solubility of KClO_3 . With nitrate, although the data at low concentrations extrapolate to the same maximum velocity as with chloride, the maximum velocity actually obtained is only 0.6 of the maximum obtained in chloride, because of the inhibition progressively

shown by nitrate in concentrations above 0.05M. On the other hand the apparent affinities are markedly different, the affinities increasing in the order: $\text{Cl}^- < \text{Br}^- < \text{NO}_3^- < \text{ClO}_3^- < \text{I}^- < \text{SCN}^-$. The more efficient activating ions are the ones which most markedly show inhibition at high concentrations. From a purely practical point of view the most efficient activators would appear to be thiocyanate or bromide.

Activity in the absence of activating anions. The stock preparation of enzyme invariably showed some activity in the absence of added NaCl, even after prolonged dialysis. The figure used as the origin of the curves in Fig. 5, i.e. an activity in the absence of salt of 0.16 of that in 0.17M-NaCl, represents the mean of nine separate experiments. Although this is quite low, it is of some interest to know whether this represents a real activity of the ion-free enzyme or an activation by anions already present in the preparation. On the assumption that this residual activity might be due to traces of firmly bound chloride in the enzyme, an attempt was made to remove it by treatment with anionic-exchange resins, both in batches and on columns. In parallel experiments De-Acidite F, Amberlite IRA-400 and Dowex 2, in acetate form, were used. There was a small increase in specific activity (which might be due to removal of inhibitors), but there was no significant change in the ratio of activity without added salt to activity in 0.17M-NaCl.

There remains the possibility that this low activity is produced by salts present in the reagents used for assay. It has been mentioned above that it is unlikely that the acetate used as a buffer has any activating power; the same activities were obtained in the absence of added buffer, when the only acetate present arose from the traces of acetic acid used in adjusting the pH of enzyme and substrate. It is possible, however, that the NPS used contained traces of chloride or other activator. Some support for this possibility is given by the fact that different values for the intrinsic activity were obtained with different batches of commercial NPS. For three batches of substrate the activities obtained in the absence of salt were 9.0, 18.5 and 11.0% respectively of those in 0.17M-NaCl. Presumably the lowest of these figures represents the maximum possible value for the intrinsic activity of anion-free enzyme.

Effect of univalent anions on enzymic hydrolysis of NCS. At an early stage in the work it was found that at no stage in the purification was there any activation of NCS hydrolysis by chloride parallel to that of NPS hydrolysis. In fact at pH 5.6 NaCl is an inhibitor of NCS hydrolysis, as shown in Fig. 6. The implications of this observation are discussed below.

Table 1. *Apparent activator constants and relative maximum velocities of activating anions*

Values obtained from intercepts of reciprocal plots of the data shown in Fig. 5; the apparent activator constant represents the concentration of activator at which half the extrapolated maximum velocity (V_{max}) is attained.

Anion	Apparent activator constant (mM)	V_{max} (relative to that in Cl^-)
Cl^-	21	1.0
Br^-	16	1.16
I^-	6	1.0
ClO_3^-	8	0.42
NO_3^-	10	1.0
SCN^-	4	1.16

Nature of the activation

In an attempt to obtain information which might throw light on the mechanism involved, the interaction of activator concentration and other variables on the enzyme activity was investigated.

pH and activation. Fig. 7 shows the activity-pH curve in 2 mM-NPS in the presence and in the absence of 0.17M-NaCl; Fig. 8 shows the corre-

sponding curves in 17 mM-NCS. It will be seen at once that the chloride activation of NPS hydrolysis cannot be explained in terms of a shift in the dissociation constants controlling the pH curve. There may be a small shift in the optimum pH from about 5.9 in the absence of chloride to 5.6 in 0.17 M-NaCl, although it is not certain that this is significant in view of the difficulty in measuring the low activities obtained in the absence of chloride. But even if the activities are compared at their

Fig. 6. Effect of chloride concentration on enzymic hydrolysis of NCS at pH 5.6. Reaction mixtures (3 ml.) contained 0.17M-acetate buffer, 83 mM-NCS and 0.06 ml. of stock enzyme. Hydrolysis rates, measured by nitro-catechol production in 10 min., are expressed relative to that without chloride.

Fig. 7. Effect of pH on enzymic hydrolysis of NPS, in the presence and in the absence of chloride. Reaction mixtures (3 ml.) contained 0.17M-acetate buffer, 2 mM-NPS and 0.2 ml. of stock enzyme. ●, No chloride; O, in 0.17M-NaCl.

Fig. 8. Effect of pH on enzymic hydrolysis of NCS, in the presence and in the absence of chloride. Reaction mixtures (3 ml.) contained 0.17M-acetate buffer, 17 mM-NCS and 0.1 ml. of stock enzyme. ●, No chloride; O, in 0.17M-NaCl.

Fig. 9. Dixon plots of data in Figs. 7 and 8. Logarithms of rates of phenol liberation by 0.1 ml. of stock enzyme are plotted against pH. ●, NPS, no chloride; O, NPS, 0.17M-NaCl; ■, NCS, no chloride; □, NCS, 0.17M-NaCl.

appropriate optimum pH, the activation by chloride is still very marked, being increased about 9.2 times by 0.17M-NaCl. In NCS hydrolysis, there is a small shift of the optimum in the same direction, from about 5.5 without chloride to 5.4 in NaCl. Both sides of the curve are shifted, so that although chloride inhibits at the optimum pH, it shows slight activating properties below pH 5.1.

Fig. 9 shows Dixon plots (Dixon, 1953; Dixon & Webb, 1958, p. 136) of the pH data. Their interpretation must be made with some reserve, since sub-optimum concentrations of substrate were used and hence true V values were not obtained; but they suggest functional groups in the enzyme with

pK values of about 5.2 and 5.9 in chloride, and perhaps rather higher in the absence of chloride.

Interaction of activator and substrate. The effect of substrate concentration on the activity towards NPS was determined in the presence and in the absence of 0.17M-NaCl; the corresponding Lineweaver-Burke plots are shown in Fig. 10. This showed that chloride has no effect on the affinity for NPS, K_m being about 4.4 mM in both. The affinity for NPS is appreciably greater than that for NCS in the absence of chloride. Fig. 11 shows K_m for NCS at pH 5.6 about 0.01 M; a similar K_m is obtained in the presence of 0.17M-NaCl.

Thus chloride appears to act by a true increase in V , i.e. in the rate of breakdown of the activated substrate at the optimum pH.

Fig. 10. Effect of chloride on the affinity of arylsulphatase for NPS at pH 5.6. Lineweaver-Burke plots; reaction mixtures (3 ml.) contained 0.17M-acetate, pH 5.6, and 0.2 ml. of stock enzyme. ●, No chloride; ○, in 0.17M-NaCl.

Fig. 11. Lineweaver-Burke plot for the hydrolysis of NCS at pH 5.6. Reaction mixtures (3 ml.) contained 0.17M-acetate buffer and 0.1 ml. of stock enzyme.

Effect of anions on the specificity of the enzyme

In characterizing arylsulphatases, use has been made by a number of authors of the specificity pattern, and in particular of the relative activity towards NPS and NCS (see Discussion). It will be clear from the results given above that the ratios obtained with the enzyme studied vary with the salt present. Thus if the activities are compared at their respective optimum pH values, but at the arbitrary substrate concentrations used in the standard tests (17 mM-NCS and 2 mM-NPS), the ratio of activities (NCS/NPS) falls from 135 in the absence of chloride to 12 in 0.17M-NaCl. If the extrapolated maximum velocities from the Michaelis curves are compared, the ratio of activity NCS/NPS at pH 5.6 is 55 in the absence of salt and 3.3 in 0.17M-NaCl.

It is, of course, desirable to establish whether these two arylsulphatase activities, differing so greatly in their response to salt, are due to a single enzyme. The ratio of activity to the two substrates does not change appreciably during the last three stages of the purification, and is not altered by further attempted fractionation by precipitation with $(\text{NH}_4)_2\text{SO}_4$ or acetone, or by adsorption on calcium phosphate gel. Further evidence of identity was obtained in two ways.

Effect of inhibitors. Fluoride and a number of multivalent anions inhibit both activities. Fig. 12 shows inhibition curves on a logarithmic scale for a number of salts for (a) NPS hydrolysis and (b) NCS hydrolysis. The inhibition by fluoride of both activities runs more or less parallel with a pI_{50} (negative logarithm of the concentration giving 50% inhibition) of 3.4. Phosphate and sulphate show the same relative activity in the two cases, but are both more effective against the hydrolysis of NPS than of NCS under the conditions studied. The pI_{50} values for phosphate and sulphate are 4.1 and 2.3 respectively against NPS hydrolysis and 3.4 and 1.6 against NCS hydrolysis. These results

would be compatible with a single enzyme hydrolysing both substrates, if phosphate and sulphate were competitive and fluoride was a non-competitive inhibitor. An attempt was made to verify this in the usual way. The results, although consistent with this possibility, were not entirely conclusive, as non-linear Lineweaver-Burke plots were obtained in the presence of inhibitors.

An alternative possible explanation of the action of the inhibitors mentioned is that they compete with the activating ion. This was ruled out by experiments in which the inhibition of NPS hydrolysis by 0.083 mM-phosphate, 6.7 mM-sulphate and 0.33 mM-fluoride was measured in the absence of NaCl and with added NaCl to a final concentration of 17 mM, 33 mM and 170 mM, in each case relative to corresponding controls without the inhibitor. For each of the inhibitors the percentage inhibition remained constant within experimental error (3–4 %); thus the apparent affinity for chloride is not affected by these inhibitors.

Hydrolysis of mixed substrates. The rate of enzyme action on a mixture of two substrates, compared with that on the two substrates present

singly, has been much used, often in a semi-qualitative way, to determine whether two reactions are due to the same enzyme. An experiment of this kind was carried out with NPS and NCS; in this instance hydrolysis was followed by the liberation of sulphate, since this is the product common to the two reactions under investigation. The results of duplicate experiments are shown in Table 2. Qualitatively the experiment suggests a single enzyme, since the mixed-substrate velocity is only about 60 % of the sum of the velocities with the substrates attacked separately.

'Mixed-substrate' experiments have most commonly been carried out with the two substrates in equimolar concentrations. In the present experiments the substrates were used at different concentrations (8.3 mM-NCS and 2.0 mM-NPS) to ensure reasonably comparable velocities; the concentrations were in fact roughly related to the Michaelis constants. The result to be expected in such a case if only one enzyme is involved is best expressed in terms of 'relative concentrations'; it can be shown (Dixon & Webb, 1958, p. 92) that

$$v_i = \frac{V_a \alpha + V_b \beta}{1 + \alpha + \beta},$$

where V_a and V_b represent the maximum velocities with excess of two substrates A and B, and v_i is the 'mixed-substrate velocity' when the relative concentrations (that is, the ratio of their concentrations to the respective Michaelis constants) of A and B are α and β . If v_a and v_b are the actual velocities when the two substrates at relative concentrations α and β are attacked singly, it can be shown, by substitution of the usual Michaelis expressions for these velocities into the equation above, that

$$v_i = \frac{v_a(1 + \alpha) + v_b(1 + \beta)}{1 + \alpha + \beta}.$$

(a)

(b)

Fig. 12. Inhibition of arylsulphatase. (a) NPS hydrolysis; (b) NCS hydrolysis. Reaction mixtures (3 ml.) contained 0.17M-acetate buffer, pH 5.6, and, for (a), 0.17M-NaCl, 6 mM-NPS and 0.2 ml. of stock enzyme; for (b), 83 mM-NCS and 0.1 ml. of stock enzyme. ○, Phosphate; ●, sulphate; □, fluoride; ■, citrate.

Table 2. Hydrolysis of p-nitrophenyl sulphate and 2-hydroxy-5-nitrophenyl sulphate by the enzyme, singly and in a mixture

Hydrolysis by 0.2 ml. of stock enzyme in 0.17M-acetate, pH 5.6, and 0.17M-NaCl, at 38°, was measured by sulphate liberated in 30 min. In each case the result is corrected for free sulphate in the substrates and any non-enzymic hydrolysis by corresponding controls without enzyme.

Concn. of substrate	Sulphate produced in 30 min. (μg.)		
	Expt. 1	Expt. 2	Mean
8.3 mM-NCS	121.5	126	124
2.0 mM-NPS	63	70.5	67
8.3 mM-NCS plus 2.0 mM-NPS	105	120	112.5

In the special case where the two substrates are mixed at the same relative concentration, i.e. $\alpha = \beta = \sigma$,

$$\frac{v_i}{v_a + v_b} = \frac{1 + \sigma}{1 + 2\sigma}.$$

Thus the mixed-substrate velocity would be the sum of the velocities with separate substrates at very low relative concentrations, but would fall to two-thirds of the sum when σ is 1 and to half the sum when σ is very high. In the present experiment the mean relative concentration of the two substrates is 0.65, which should give a value of $v_i/(v_a + v_b)$ of 0.71. The experimental value of 0.6 is in reasonable agreement with this, considering the difficulty of the benzidine sulphate-assay method for sulphatase activity. It seems highly probable that a single enzyme in the preparation studied hydrolyses both NPS and NCS.

DISCUSSION

Although many enzymes are activated by cations, activation by anions is comparatively rare. The classical case is perhaps the activation of animal α -amylase by chloride discovered by Cole (1904) and investigated in detail by Myrbäck (1926). A number of univalent anions were effective but chloride was most efficient, the order of effectiveness being $\text{ClO}_3^- < \text{NO}_3^- < \text{I}^- < \text{Br}^- < \text{Cl}^-$. This is almost the reverse of the order of activity found with arylsulphatase. Another well-known case is that of fumarase, studied by Mann & Woolf (1930) and Massey (1953). For this enzyme multivalent ions were effective, arsenate being most active and phosphate, citrate, borate, arsenite, selenate and sulphate progressively less so; chloride was inhibitory. For both fumarase and α -amylase the effects of anions are readily seen, from the pH-activity curves, to be due to an effect on the dissociation of the ionizing groups at the active centre of the enzyme, which determine the optimum pH. The more effective activators for both enzymes raise the pK of the basic group involved but have little effect on the acidic group; with some other ions both pK values are raised so that the whole curve is displaced, producing an inhibition on the acid side and an activation on the alkaline side but little change in the maximum velocity. It seems probable from the pH curves presented in this paper that the activation of arylsulphatase is not of this type.

Another mechanism whereby ions might affect enzyme activity is by influencing the combination of enzyme and substrate. Theorell and his co-workers (Theorell, Nygaard & Bonnichsen, 1955; Theorell, 1958) found that, with liver alcohol dehydrogenase, chloride reduces the affinity for

reduced coenzyme I by increasing the rate of dissociation from the enzyme, but decreases the rate of dissociation of the oxidized coenzyme-enzyme complex. Thus chloride can act as either an activator or an inhibitor of alcohol dehydrogenase according to the experimental conditions. Chloride has no effect on the affinity of arylsulphatase for either of the substrates studied, and it seems probable that we are dealing here with a direct effect of chloride on the breakdown of the enzyme-substrate complexes; no clear case of such an effect of anions has hitherto been established.

The relative rates of hydrolysis of various derivatives of phenyl hydrogen sulphate, and in particular NCS and NPS, have been much used recently as a criterion for distinguishing different arylsulphatases. Dodgson & Spencer (1957) proposed that arylsulphatases should be divided into two groups: type I enzymes, showing considerable affinity for and activity towards simple arylsulphatases such as NPS and *p*-acetylphenyl sulphate, but much less activity towards NCS, and being strongly inhibited by cyanide but unaffected by sulphate or phosphate; and type II enzymes, showing little activity towards NPS but considerably more towards disubstituted phenyl sulphates such as NCS, and being strongly inhibited by phosphate and sulphate but not cyanide. Of the three arylsulphatases from animal liver previously described, arylsulphatase C is a type I enzyme, and arylsulphatases A and B are type II enzymes. The last two enzymes were originally distinguished by Roy (1953*a*) by their action on NCS; arylsulphatase A shows an optimum pH of 4.9 and an optimum substrate concentration of 3 mM-NCS, whereas arylsulphatase B shows an optimum pH of 6.0 (or 5.7 in chloride) and an optimum substrate concentration greater than 0.03M. Roy (1958), in comparative studies of the distribution of these enzymes in the livers of a number of species, used NPS and NCS as diagnostic substrates for arylsulphatase C and arylsulphatases A and B respectively. It is obvious that such criteria must be treated with great caution unless the ionic composition of the reaction medium is rigorously defined, in view of the possible profound effect of anions on the specificity pattern and optimum pH. Caution is particularly necessary when dealing with crude preparations which may contain appreciable amounts of chloride extracted from the tissue used as a source of enzyme.

The relationship of the enzyme described in this paper to arylsulphatases A, B and C of ox liver is not completely clear. The properties of these three enzymes have been tabulated by Roy (1956). The characteristics of the present enzyme agree most closely with those of arylsulphatase B, especially in optimum pH for NCS hydrolysis, inhibition by

phosphate, sulphate and fluoride and insensitivity to cyanide. The affinities for both NPS and NCS are, however, considerably greater than those reported for arylsulphatase B and are, in fact, close to those given for arylsulphatase C (which, however, is insoluble and sensitive to cyanide but not to sulphate). The NCS/NPS activity ratios are given by Roy as 20 for A, > 50 for B and 0.5 for C [Dodgson & Wynn (1958) give 17 for human-liver arylsulphatase B]; all these values were obtained at low chloride concentration. The enzyme described here gives an NCS/NPS activity ratio of 55 in the absence of salt and 3.3 in chloride; this seems reasonably compatible with its identity with arylsulphatase B.

Other enzymes from animal tissues hydrolysing aromatic substances have been reported with properties not unlike those of arylsulphatase; for example Singer & Fruton (1957) showed that the same enzyme from spleen probably hydrolysed phenyl phosphates, phosphoramidate and phosphoprotein; this enzyme had an optimum pH near 6 and was inhibited by phosphate and fluoride. The relationship between the hydrolytic activities of animal tissues towards various synthetic substrates containing aryl groups is being investigated.

SUMMARY

1. The partial purification of an enzyme in ox liver hydrolysing *p*-nitrophenyl sulphate (NPS) and 2-hydroxy-5-nitrophenyl sulphate (NCS) is described.

2. The use of the Ratio Recording Spectrophotometer for following the progress of NPS hydrolysis is described.

3. The hydrolysis of NPS by the enzyme is strongly activated by chloride and some other univalent anions whereas the hydrolysis of NCS by the enzyme is inhibited by chloride.

4. The apparent affinities for activating anions, as observed by NPS hydrolysis, increase in the order Cl^- , Br^- , NO_3^- , ClO_3^- , I^- , SCN^- . The maximum velocity obtained with each ion except ClO_3^- is almost the same; but the velocity falls off at higher concentrations of thiocyanate, nitrate and iodide.

5. The activity of the enzyme towards NPS in the absence of added salts could not be reduced below 9% of that in 0.17M-NaCl.

6. Chloride has little effect on the dependence of either NPS or NCS hydrolysis on pH.

7. Chloride has no effect on the affinity of the enzyme for either NPS or NCS.

8. Phosphate, sulphate and fluoride inhibit the action of the enzyme on both NPS and NCS; the effect is not due to competition with activator, but phosphate and sulphate may compete with substrate.

9. Mixed-substrate experiments suggest that both substrates are attacked by a single enzyme in the preparation.

10. The implications of this work for the characterization of sulphatases and the relationship of the present enzyme to the arylsulphatases previously described are discussed.

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