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Studies on Sulphatases

19. THE PURIFICATION AND PROPERTIES OF ARYLSULPHATASE B OF HUMAN LIVER*

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Evidence is accumulating which suggests that two different types of arylsulphatases occur in Nature. Dodgson (1956) and Dodgson & Spencer (1957*a*) have tentatively suggested that those arylsulphatases which show considerable affinity and activity towards simple arylsulphates such as potassium *p*-nitrophenyl sulphate (NPS) and potassium *p*-acetylphenyl sulphate (APS) should be classified as type I arylsulphatases. This type of enzyme is strongly inhibited by cyanide but is not appreciably affected by sulphate or phosphate. On the other hand, those enzymes which show relatively little affinity and activity towards NPS and APS but considerable affinity and activity towards the more complex arylsulphate, dipotassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate, NCS), are classified as type II arylsulphatases. These enzymes are strongly inhibited by sulphate and phosphate but are unaffected by cyanide. Considerable information on the mode of action of one example of a type I enzyme (the bacterial arylsulphatase from *Alcaligenes metalcaligenes*) has already been obtained (Dodgson, Spencer & Williams, 1955, 1956*a*).

Examples of both types of arylsulphatases occur in human liver and other tissues (Dodgson, Spencer & Wynn, 1956*b*). Arylsulphatase C, a type I enzyme, is associated with the microsomes of the liver cell and has not yet been obtained in soluble form. Arylsulphatases A and B are type II enzymes and can readily be obtained in soluble form. The present communication describes the purification and properties of human-liver arylsulphatase B and gives some indication of the nature of the ionizing groups which are involved in the enzymic reaction. A preliminary account of this work has already appeared (Wynn & Dodgson, 1957).

* Part 18: Dodgson & Lloyd (1957).

MATERIALS AND METHODS

Substrates. APS, NPS and potassium phenyl sulphate were prepared by the method of Burkhardt & Lapworth (1926), NCS by the method of Roy (1953) as modified by Dodgson & Spencer (1956*a*) and the monopotassium salts of 4-hydroxy-2-nitrophenyl and 4-hydroxy-3-nitrophenyl sulphates by the method of Smith (1951). The monopotassium salts of *o*-hydroxyphenyl sulphate (catechol monosulphate) and 4-chloro-2-hydroxyphenyl sulphate (4-chlorocatechol monosulphate) were prepared from catechol and 4-chlorocatechol respectively by the method of Burkhardt & Lapworth (1926), but sufficient chlorosulphonic acid was used to sulphate one hydroxyl group only (cf. Dodgson, Rose & Spencer, 1955). Tyrosine *O*-sulphate was prepared by the method of Tallan, Bella, Stein & Moore (1955).

Buffers. In most of the enzyme experiments sodium acetate–acetic acid mixtures (hereafter referred to as acetate buffers) were used to control pH. However, in certain experiments where the properties of the human enzyme were being compared with those of the corresponding enzyme of ox liver, substrate (NCS) solutions were buffered with the acetate–HCl mixture (hereafter referred to as acetate–HCl buffer) described by Roy (1953, 1954). In preparing these solutions Roy used HCl to overcome the strong buffering action of the substrate, but when substrate solutions of varying concentration were required it automatically followed that different amounts of HCl had to be introduced in order to achieve the same pH.

Determination of enzyme activity. Enzyme activity towards APS, NPS and NCS was measured by spectrophotometric estimation of the respective liberated phenols (Dodgson, Spencer & Thomas, 1955). A similar method was

used to determine activity towards 4-hydroxy-2-nitrophenyl and 4-hydroxy-3-nitrophenyl sulphates, the anionic forms of the liberated phenols being estimated spectrophotometrically at a wavelength of 540 mμ. Enzyme activity towards all other substrates was determined by estimating liberated sulphate by the method of Dodgson & Spencer (1953). Except where otherwise stated, incubation of enzyme and substrate was for 1 hr. at 37·5° in the presence of 0·5 M-acetate buffer adjusted to the appropriate pH. One unit of arylsulphatase B activity is defined as that which liberates 1 μg. of nitrocatechol in 1 hr. at 37·5° from 0·01 M-NCS in the presence of 0·5 M-acetate buffer, pH 6·1.

Determination of protein. The method of Lowry, Rosebrough, Farr & Randall (1951) was used. A calibration curve was constructed with varying concentrations of the stage 3 enzyme preparation and spectrophotometric readings were made at a wavelength of 750 mμ.

Determination of nucleic acid. The ratio of the spectrophotometric readings at 280 and 260 mμ was used to determine nucleic acid (Warburg & Christian, 1941).

EXPERIMENTAL AND RESULTS

Preliminary purification of arylsulphatase B

Post-mortem samples of non-pathological human livers were obtained within 48 hr. after death. Acetone-dried powders of these livers were made according to the directions of Dodgson, Rose, Spencer & Thomas (1957).

Stage 1. Acetone-dried liver (60 g.) was suspended (Townson & Mercer top-drive macerater) in 500 ml. of 0·1 M-sodium acetate solution which had been adjusted to pH 7 with a few drops of acetic acid. After incubating at 37·5° for 30 min. the suspension was cooled to 0° and clarified by centrifuging at 6000 *g* and 0° for 30 min. The supernatant was kept at 0° whilst the debris was washed by resuspending it in a further 300 ml. of the acetate mixture and centrifuging as before. The two supernatants (approx. 700 ml.) were combined and diluted with sufficient water (usually about 300 ml.) to give a final protein concentration of 0·5 %.

Stage 2. The enzyme solution was cooled to 0° and acetone (at 0°) was added slowly with stirring until the concentration of acetone was 40 % (v/v). The whole was allowed to stand at −5° for 20 min. and the precipitate was separated by centrifuging at −5° and suspended in a volume of water equal to the volume of acetone originally added. After dialysing overnight against running water the suspension was clarified by centrifuging at 25 000 *g* and 0° for 30 min., and the clear supernatant solution diluted with water to give a final protein concentration of 0·25 %.

Up to this stage in the purification procedure it was not possible to determine the activity of arylsulphatase B with certainty owing to the presence of arylsulphatase A in the enzyme preparation. However, most of the latter enzyme is eliminated during stage 2 (cf. Dodgson *et al.* 1956*b*). The arylsulphatase B activity of the final solution was approx. 200 units/mg. of protein.

Stage 3. Sufficient sodium acetate and $CaCl_2$ were added to the enzyme solution to give final concentrations of 0·03 and 0·003 M respectively and the pH was adjusted to 6·5 with a few drops of acetic acid. After cooling to 0° the solution was treated with acetone at 0° until the concentration of acetone was 30 % (v/v), and after standing at −5° for 30 min. the precipitate was separated by centrifuging at 6000 *g* and −5° for 20 min. The precipitate was dissolved in water (150 ml.) before dialysing for 24 hr. against running water. The cloudy solution was clarified by centrifuging at 25 000 *g* and 0° for 30 min. The arylsulphatase B activity of the final clear solution was approx. 400 units/mg. of protein and the total yield of enzyme was 60 % of that present at stage 2.

Stage 4. The enzyme solution (approx. 175 ml.) was dialysed at room temperature for 3 days against several changes of distilled water (a total of 15 l./50 ml. of enzyme solution). The precipitate which appeared was separated by centrifuging at 25 000 *g* and 0° for 30 min. The bulk of the enzyme activity was present in this precipitate which was then allowed to stand in the presence of M-sodium acetate (20 ml./50 ml. of the stage 3 preparation) at 0° for 24 hr. This treatment dissolved most of the arylsulphatase B, the final enzyme solution being clarified by centrifuging as before. The arylsulphatase B activity of this preparation was approx. 800 units/mg. of protein; yield, 40 % of stage 3.

Properties of the stage 4 *enzyme.* Typical substrate concentration–activity and pH–activity curves for the stage 4 enzyme are shown in Figs. 1 and 2 respectively. Both curves exhibited marked anomalies which varied from preparation to preparation. Further investigations showed that enzyme activity could be markedly affected by varying the concentration of acetate buffer in the incubation mixture (Fig. 3). It seemed possible that the presence of small amounts of arylsulphatase A in the preparation might be responsible for these anomalies, but paper electrophoresis of the preparation under the conditions described by Dodgson & Spencer (1956*b*) gave no evidence of the presence of this enzyme. Moreover, Dodgson & Spencer (1956*a*, *c*) have shown that human arylsulphatase A gives an anomalous time–activity curve when assayed against 0·005 M-NCS in the presence of 0·5 M-acetate buffer, pH 5. The time–activity curve of the stage 4 enzyme preparation, when measured under these conditions, was linear and showed no anomalies. It was therefore tentatively assumed that the anomalies shown by arylsulphatase B were due to the varying tendency of the enzyme protein to combine with inert

protein under the varying conditions of the enzyme experiments and attempts were therefore made to purify the enzyme further.

Further purification of the enzyme

Stage 5. The stage 3 enzyme preparation was used as starting material for further purification. The enzyme solution (usually in 80 ml. portions) was adjusted to pH 7 and an aqueous solution of protamine sulphate (0·1 %), which had been adjusted to pH 8 with N-NaOH, was added slowly until the clear supernatant obtained by centrifuging at 25 000 *g* and 0° for 5 min. no longer gave a precipitate on adding further protamine sulphate solution. The final clear supernatant was adjusted to pH 9 with N-NaOH, the precipitate removed by centrifuging as before and the supernatant was dialysed for 12 hr. at 5° against distilled water (100 vol.). After clarifying by centrifuging, the arylsulphatase B activity of the solution was approx. 1000 units/mg. of protein. Yield, 90 % of stage 3. The concentration of nucleic acid in the enzyme preparation was reduced from approx. 0·8 % to zero during this procedure.

Stage 6. Sufficient sodium acetate was added to the enzyme solution to give a final concentration of 0·1 M and the whole was adjusted to pH 6 with acetic acid. This solution was allowed to filter by gravity at room temperature through dry grade 4 sintered-glass funnels (no more than 1000 units of activity/funnel) which had previously been cleaned with boiling nitric acid followed by boiling water. The diameter of the sintered-glass disk of each funnel was approx. 30 mm. An appreciable amount of inert protein passed through the sintered disks which, however, retained a considerable part of the arylsulphatase activity. Each sintered disk was washed (by gravity) with 2 ml. of 0·1 M-acetate buffer, pH 6, and the enzyme was subsequently eluted from the disk with 3 ml. of M-acetate buffer, pH 6. The arylsulphatase B activity of the final

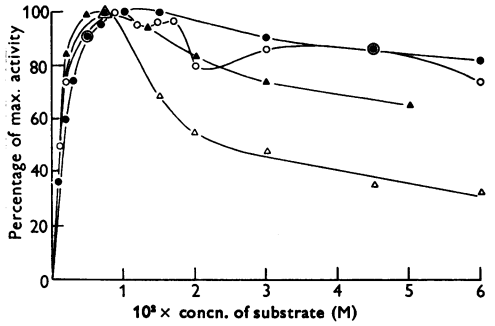


Fig. 1. Substrate concentration–activity curves for human arylsulphatase B acting on NCS. Incubation was for 1 hr. at 37·5°. ○, Stage 4 enzyme incubated in the presence of 0·5 M-acetate buffer, pH 6·1; ●, stage 6 enzyme in the same buffer; △, stage 4 enzyme or stage 6 enzyme incubated with substrate solutions buffered at pH 5·7 with acetate–HCl according to the directions of Roy (1954); ▲, stage 6 enzyme incubated in the presence of 0·5 M-acetate buffer, pH 5·7. The curves are not quantitatively related to each other.

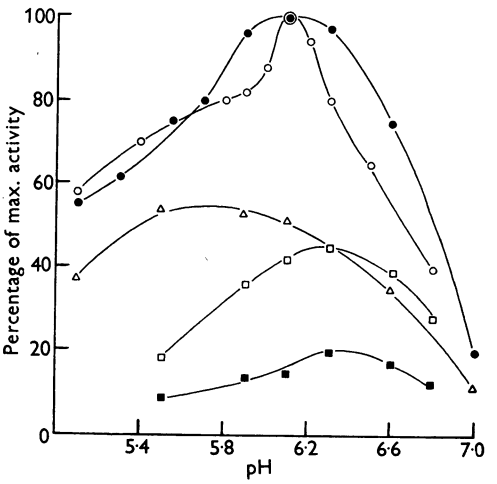


Fig. 2. pH–Activity curves for arylsulphatase B of human liver. The final concentration of substrate (NCS) was 0·01 M and incubation was for 1 hr. at 37·5° in the presence of 0·5 M-acetate buffer. ○, Stage 4 enzyme; ●, stage 6 enzyme; ■, stage 6 enzyme in the presence of 0·0025 M-NaH_2PO_4; □, stage 6 enzyme in the presence of 0·1 M-Na_2SO_4; △, stage 6 enzyme in the presence of 0·08 M-KCl. Stage 6 enzyme curves are quantitatively related to each other.

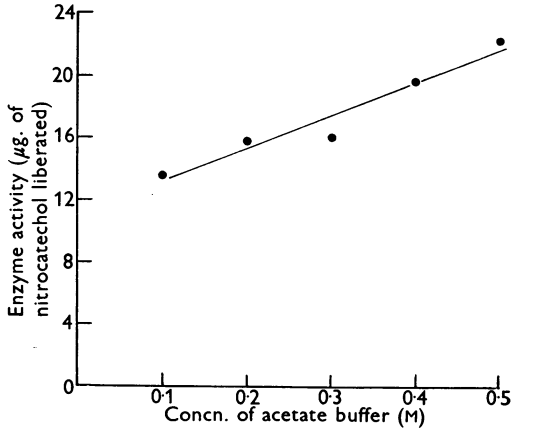


Fig. 3. Effect of buffer concentration on the activity of human arylsulphatase B at stage 4 in the purification procedure. The final concentration of substrate was 0·01 M-NCS and incubation was for 1 hr. at 37·5° in the presence of varying concentrations of acetate buffer, pH 6·1.

solution was approx. 3000 units/mg. of protein. Yield, 40% of stage 5. Examination of the various filtrates and the original enzyme solution by phase-contrast microscopy suggested that true adsorption of enzyme on glass rather than a simple filtration of particulate enzyme was involved. This was confirmed by the finding that the enzyme was also strongly adsorbed on powdered glass and on Ballotini no. 12 glass beads (English Glass Co. Ltd., Leicester).

Properties of the purified enzyme

Substrate concentration–activity and pH–activity curves for the purified enzyme are shown in Figs. 1 and 2 respectively. The curves no longer exhibited the anomalies shown by the stage 4 enzyme preparation, although the pH–activity curve was still asymmetric. Acetate concentration no longer affected the activity of the enzyme over the concentration range studied (0·1–1 M).

The behaviour of the enzyme towards increasing concentrations of substrate (optimum substrate concentration of 0·01 M-NCS in 0·5 M-acetate buffer, pH 6·1) contrasts sharply with that of the partially purified corresponding enzyme of ox liver. With the latter Roy (1954) observed that, at the optimum pH (5·7), arylsulphatase activity continued to increase with increasing substrate concentration and failed to reach a maximum even at the practical limit of substrate solubility (0·06 M-NCS). However, as mentioned earlier, the method used by Roy (1954) to prepare substrate solutions was such that an increase in the concentration of NCS was paralleled by an increase in the concentration of Cl^- ions. Exact repetition of Roy's work with the ox enzyme has confirmed his observations (see Fig. 4). However, when Cl^- ions were omitted from the incubation mixtures a considerable change in the substrate concentration–activity curve was obtained. Moreover, the shape of the curve was markedly affected by the concentration of the acetate buffer used (Fig. 4). Since activity of the human enzyme was also affected by acetate concentration during the early stages of purification, the ox enzyme was purified further (× 15) by treatment with protamine sulphate and adsorption on sintered-glass disks as described earlier for the human enzyme. Although the activities of the two enzymes/mg. of protein were now approximately comparable the ox enzyme was still affected by acetate concentration and the substrate concentration–activity curve, when determined in the presence of increasing concentrations of Cl^- ions at pH 5·7, was similar to that obtained before further purification. Under identical experimental conditions (i.e. pH 5·7 in acetate–HCl buffer) both the stage 4 and stage 6 preparations of the human enzyme gave substrate concentration–activity curves which deviated from the normal curve obtained at this pH only by virtue of an apparent increase in the degree of inhibition by excess of substrate (see Fig. 1). However, this increase can probably be attributed to the inhibiting effect of Cl^- ions, substrate solutions of higher concentration containing greater amounts of these ions. Fig. 2 shows the effect of Cl^- ions (0·08 M-KCl) on the pH–activity curve of the human enzyme. Considerable inhibition occurs under these conditions and there is a shift in the pH optimum in the direction of lower pH. Roy (1955) has observed a similar shift in the pH optimum of the ox enzyme when determined in the presence of 0·08 M-KCl, but this was accompanied by an appreciable increase in the activity of the enzyme. It is possible therefore that the anomalous substrate concentration–activity curve obtained for the ox enzyme when acetate–HCl buffer is used is at least partly due to the activating effect of the increasing amounts of Cl^- ions which parallel the increases in substrate concentration.

In certain other respects the two enzymes exhibit similar behaviour. Fig. 2 shows the effect of $H_2PO_4^-$ and SO_4^{2-} on the pH–activity curve of the stage 6 human enzyme. Considerable inhibition occurs with both ions, the resultant curves being very similar to those obtained by Roy (1955) for the ox enzyme.

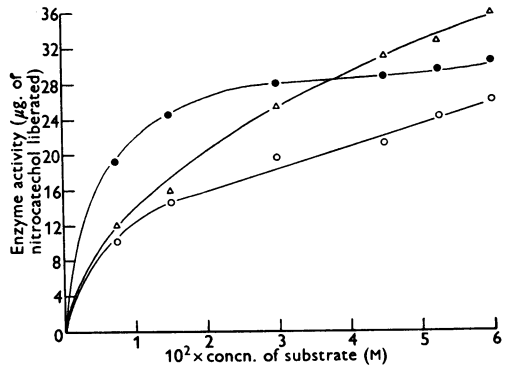


Fig. 4. Substrate concentration–activity curves for the arylsulphatase B of ox liver acting on NCS. Incubation was for 1 hr. at 37·5°. ○, Ox arylsulphatase B prepared according to the directions of Roy (1954) and incubated in the presence of 0·1 M-acetate buffer, pH 5·7; ●, the same concentration of enzyme incubated in the presence of 0·5 M-acetate buffer, pH 5·7; △, the same concentration of enzyme incubated with substrate solutions buffered at pH 5·7 with acetate–HCl according to the directions of Roy (1954), and which therefore contained varying amounts of Cl^- ions.

Nature of the dissociating groups involved in the enzyme reaction

In the experiments described below, Michaelis constants (K_m) and maximum velocities

$$(V_{max.} = k_3[E])$$

were determined by the method of Lineweaver & Burk (1934), with case III, which takes into account any inhibition by excess of substrate. Values of $V_{max.}$ are relative values expressed in arbitrary units since, although the concentration of enzyme used was the same in all experiments, the absolute concentration [E] was unknown.

Variation of K_m with pH. A number of workers have shown that information regarding the functional groups of enzymes can be obtained by consideration of the variation of the dissociation constant (K_s) of the enzyme–substrate complex with pH (see Bray & White, 1957), and Dixon (1953) has formulated certain rules which govern the interpretation of these variations. Under certain circumstances when, as in the present case, direct determination of K_s is not possible, these rules can be applied to the variation of the Michaelis constant (K_m) with pH. These circumstances have previously been discussed by Dodgson *et al.* (1955) and will not therefore be reiterated. It suffices to say that Dixon (1953) states that rules 9 and 10 can be applied to the pK_m (p$K_m = -\log K_m$)–pH curve in those regions where the pV

$$(pV = -\log V_{max.})\text{–pH}$$

curve is either (1) horizontal or (2) has non-whole-number slopes or is curved, provided that, in the second case, the pK_m–pH curve has whole-number slopes (e.g. 0- or 1-unit slopes) in the same pH regions.

Fig. 5 shows the pK_m–pH and pV–pH curves for arylsulphatase B acting on NCS and it may be deduced from these curves that Dixon's rules are applicable in the present case. It can be seen from Fig. 5 that there is a change in direction of the pK_m–pH curve in the region of pH 5·7. According to Dixon's rule 10 this change in direction reflects the pK value of an ionizing substrate grouping or a substrate-binding enzyme grouping. The substrate contains no ionizing group with pK in the region of 5·7 (the pK of the free phenolic group of NCS is 6·5; Dodgson *et al.* 1955) and the change in direction at this pH may therefore be presumed to be due to the ionization of a substrate-binding enzyme group.

Further information may be derived from the pK_m–pH curve by considering Dixon's rule 9, which states that the slope in any region of the curve is numerically equal to the change of charge on...'desubstration', that is to say, it is equal to the number of charges on the left-hand side of the equation

$$E + S \rightleftharpoons ES \qquad (1)$$

minus the number of charges on the right-hand side, due regard being paid to algebraic sign. Since there is no evidence from the pK_m–pH curve of change in charge of enzyme–substrate complex or of substrate over the pH range studied it follows that the enzyme group with pK 5·7 is of a type which gains a negative charge or loses a positive charge above pH 5·7 to give a -1 unit slope.

Variation of $V_{max.}$ with pH. Fig. 6 shows the variation of the maximum velocity ($V_{max.} = k_3[E]$) with pH. From Figs. 5 and 6 it can be seen that whereas, over the range pH 5–5·7, K_m remained constant there was a significant increase in $V_{max.}$.

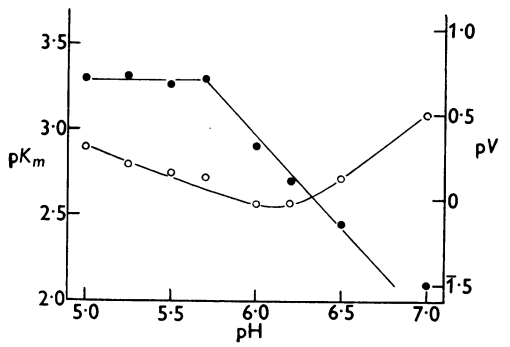


Fig. 5. Variation with pH of pK_m ($=\log K_m$) and pV ($=\log V_{max.}$) for human arylsulphatase B acting on NCS. Incubation was for 1 hr. at 37·5° in the presence of 0·5 M-acetate buffer. ●, pK_m; ○, pV.

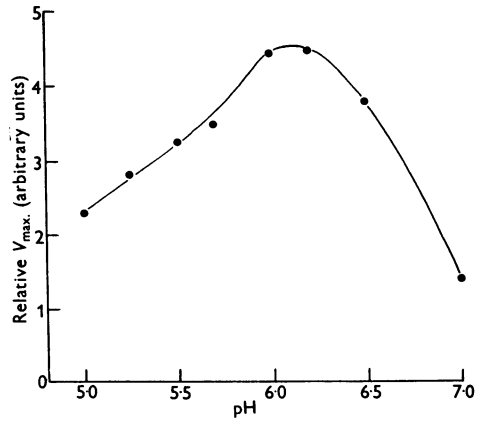


Fig. 6. Variation with pH of the maximum velocity ($V_{max.}$) of the hydrolysis of NCS by human arylsulphatase B. Incubation was for 1 hr. at 37·5° in the presence of 0·5 M-acetate buffer. The values of $V_{max.}$ are relative values expressed in arbitrary units.

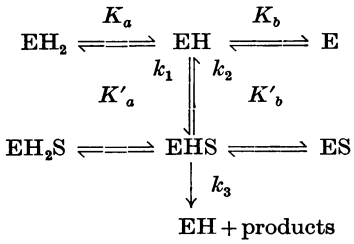
Similar findings have been made for uricase (Baum, Hübscher & Mahler, 1956) and for chymotrypsin acting on acetyl-L-phenylalanine ethyl ester (Hammond & Gutfreund, 1955). It must be assumed that the reaction at the specific substrate-binding site of the enzyme, resulting in the formation of the enzyme–substrate complex, is independent of the subsequent reaction of the substrate with the catalytic site(s) of the enzyme.

It has been pointed out (Botts & Morales, 1953) that changes in $V_{max.}$ which occur on the acid side of the pH optimum of an enzyme can be attributed to non-competitive inhibition of a catalytic site of the enzyme by H^+ ions. In such a case the ionization constant of the catalytic site is synonymous with the inhibitor constant, $K_i=[E][H^+]/[EH^+]$, where $[E]$ is the concentration of active enzyme and $[EH^+]$ is the concentration of inhibited enzyme. Hammond & Gutfreund (1955) have pointed out that, from the equations given by Lineweaver & Burk (1934), the following relationship can be derived between inhibitor concentration $[H^+]$, $k_3[E]$ (the maximum velocity at a particular concentration of $[H^+]$) and $k_3[E_0]$ (the maximum velocity when all the enzyme is present in the active form):

$$[E_0]/[E]=1+[H^+]/K_i. \qquad (2)$$

K_i may be calculated from the slope $(1/K_i)$ of the curve obtained by plotting $[E_0]/[E]$ against $[H^+]$. Fig. 7 shows the plot of $[E_0]/[E]$ against $[H^+]$ for arylsulphatase B, the ratio $[E_0]/[E]$ for the different values of $[H^+]$ being calculated from determinations of $k_3[E]$, assuming $k_3[E_0]=k_3[E]$ at pH 6·1. The value for K_i (and hence the ionization constant of the catalytic site) obtained from this graph is 7×10^{-6} and pK_i $(-\log K_i)=5{\cdot}2$.

Further information regarding the nature of the catalytic sites present in the enzyme–substrate complex can be obtained from the $V_{max.}$–pH curve (Fig. 6). Michaelis & Davidsohn (1911) and Michaelis & Pechstein (1914) suggested that the bell-shaped curve which is generally obtained when $V_{max.}$ is plotted against pH is the result of the ionization of acidic and basic groups of the enzyme. On the basis of this concept, Alberty & Massey (1954) suggested that the reaction mechanism (3) could apply to such a system.



(3)

In reaction (3) EH is the catalytically active form of the enzyme and K_a and K_b are acid dissociation constants for the two groups. By steady-state treatment these workers derived the following expression for the maximum velocity:

$$V_{max.}=\frac{k_3[E_0]}{1+[H]/K'_a+K'_b/[H]}. \qquad (4)$$

If the symbol $V_{(max.)}$ be allowed to represent the highest maximum velocity obtained at a H^+ ion concentration $H_{max.}$ (i.e. the highest point on the $V_{max.}$–pH curve) then it is possible to derive from this expression the following equations:

$$K'_aK'_b=(H_{max.})^2, \qquad (5)$$

$$K'_a=H_a+H_b-4H_{max.}, \qquad (6)$$

where H_a is the H^+ ion concentration at which the value $V_{max.}/V_{(max.)}$ on the acid side of $H_{max.}$ is equal to 1/2 and H_b is the H^+ ion concentration at the corresponding point on the basic side (Alberty & Massey, 1954). K'_a and K'_b for arylsulphatase B were 5·3 and 6·9 respectively. The value 5·3 is in good agreement with the value (5·2) obtained by the method of Hammond & Gutfreund (1955). To summarize, there are present in the enzyme–substrate complex two ionizing groups with pK in the region of 5·3 and 6·9 respectively, the ionization of which affects the breakdown of the enzyme–substrate complex.

Asymmetric nature of the $V_{max.}$–pH *curve.* It will be noted from Fig. 6 that the $V_{max.}$–pH curve decreases more sharply on the basic side of the maximum than on the acidic side. Alberty & Massey (1954) have pointed out that such asymmetric curves could occur if EH_2S or ES (see eqn. 3) or both were also able to break down to yield product, though at slower rates than EHS. They argue that the fact that zero activity is generally

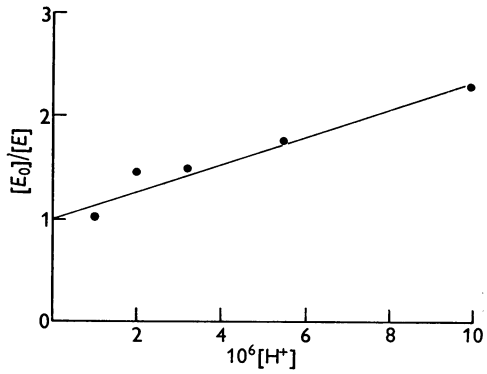


Fig. 7. Effect of H^+-ion concentration on the maximum velocity of the hydrolysis of NCS by human arylsulphatase B. The curve is plotted according to the method of Hammond & Gutfreund, 1955 (see text).

approached in solutions of increasing acidity or basicity indicates that such reactions may be ignored. However, the reaction mechanism proposed by these workers (eqn. 3) assumes that the substrate remains in the same state of ionization throughout the pH range studied. In the case of arylsulphatase B the free phenolic group of the substrate (NCS) is known to dissociate (pK 6·5) and in such a case it becomes necessary to consider a system in which the substrate, in addition to the enzyme, can exist in more than one ionic form. Laidler (1955*a*, *b*) has considered systems of this type from a theoretical point of view and has pointed out that ionization of substrate could affect the symmetry of their $V_{max.}$–pH curves. In the present case there is no reason to suppose that the pK of the free phenolic group of the substrate is appreciably altered when present in the enzyme–substrate complex, and it seems possible that increasing ionization of this group on the basic side of the $V_{max.}$–pH curve could be responsible for the sharper decline in enzyme activity which occurs on this side of the curve.

Activity of the enzyme towards other arylsulphates

The activity of the enzyme towards certain other arylsulphates was tested under arbitrary experimental conditions. The results are shown in Table 1, from which it can be seen that, under the conditions used, the enzyme showed little activity towards potassium phenyl sulphate and its monosubstituted derivatives. On the other hand, appreciable activity towards the disubstituted derivatives was obtained. A systematic study is now being made in order to establish more clearly the effect of molecular configuration of substrate on enzyme activity.

Table 1. *Relative activity of human arylsulphatase B towards the potassium salts of certain arylsulphates*

The stage 6 enzyme preparation was used and the final concentration of each substrate was 0·0125 M in 0·5 M-acetate buffer, pH 6·1. Incubation was for 1 hr. at 37·5°.

Arylsulphate	Arylsulphatase activity (μg. of SO_4^{2-} ion liberated/hr.)
2-Hydroxy-5-nitrophenyl sulphate	30
4-Hydroxy-3-nitrophenyl sulphate	34
4-Hydroxy-2-nitrophenyl sulphate	5
4-Chloro-2-hydroxyphenyl sulphate	10
o-Hydroxyphenyl sulphate	0
Phenyl sulphate	0
p-Acetylphenyl sulphate	0
p-Nitrophenyl sulphate	2
Tyrosine *O*-sulphate	0

DISCUSSION

The purification procedure described for human arylsulphatase B has been successfully applied to a large number of different samples of human liver. It should be noted, however, that it is important to test the liver samples for arylsulphatase activity towards NCS before purification since occasionally livers have been obtained which possessed negligible activity. On two other occasions the purification procedure has failed to work satisfactorily. No attempts have been made to investigate these points fully but the explanation probably lies in the variation in the degree of degenerative change occurring in the liver between the times of death and the post-mortem sampling.

The reasons for the differences observed between the ox and human enzymes are not yet clear. They may be fundamental in character or they may merely reflect degenerative changes which may have taken place in the human liver and which would be minimal in the fresh ox-liver samples. Such changes might result in modification of the enzyme or of other protein constituents of the liver with which the enzyme is associated. In this connexion it is worth noting that, irrespective of the degree of purification, the human-liver enzyme has never given an anomalous substrate concentration–activity curve corresponding to that obtained for the ox enzyme (see also Dodgson *et al.* 1956*b*). On the other hand, Dodgson & Spencer (1957*b*) obtained a substrate concentration–activity curve for the arylsulphatase activity of human serum which was still ascending at a concentration of 0·06 M-NCS and which closely resembled that obtained for the ox-liver enzyme buffered with acetate–HCl.

The investigations on the mechanism of action of human arylsulphatase B have indicated that there is present in the enzyme an ionizing group with pK about 5·7 which is at least partly responsible for the binding of substrate. The group loses a positive charge (or gains a negative charge) above pH 5·7 but its chemical nature is at present obscure. The reaction of substrate with this group, resulting in the formation of the enzyme–substrate complex, is apparently independent of the subsequent reaction of the substrate with the catalytic site(s) of the enzyme. Not only does the maximum velocity of the enzyme reaction increase appreciably over the pH range (5·0–5·7) where the affinity of enzyme for substrate remains constant but it continues to increase between pH 5·7 and 6·1 where enzyme–substrate affinity is decreasing sharply.

It has also been shown that there are present in the enzyme–substrate complex two groups, the ionizations of which affect the breakdown of the

complex. The pK of one of these groups has been determined by two different methods as being about 5·2. It is questionable, however, whether the pK 6·9 obtained for the second group can be regarded as significant in view of the asymmetric nature of the $V_{max.}$–pH curve from which it was calculated. If ionization of the free phenolic group of the substrate is responsible for the fact that this curve decreases more sharply on the basic side of the maximum than on the acid side then the value pK 6·9 calculated for the basic catalytic site of the enzyme will be a composite value of the pK values of the phenolic substrate group and the basic catalytic site when both are present in the form of enzyme–substrate complex.

It is already clear that the mechanism of action of human arylsulphatase B (a type II arylsulphatase) differs in one respect from that of the arylsulphatase of *Alcaligenes metalcaligenes* (a type I enzyme). With the latter enzyme no indications were obtained that the binding of substrate by the enzyme was independent of the subsequent reaction of substrate with the catalytic site(s) of the enzyme since there was no significant change in $V_{max.}$ over the pH range where K_m remained constant (see Dodgson *et al.* 1955). It will now be important to examine other examples of both types of arylsulphatase in order to establish whether difference in reaction mechanism is the fundamental reason for the differences observed in the properties of the two types of enzyme.

The meagre evidence available at present suggests that the activity of arylsulphatase B is greatly enhanced by the presence in the substrate of a free phenolic group in addition to a second substituent group. In the absence of this free phenolic substrate group the enzyme is only feebly active towards arylsulphates. If ionization of the free phenolic group of NCS is a contributory factor to the sharp decrease in the $V_{max.}$–pH curve which occurs on the basic side of the maximum, the further implication is that maximum activity of the enzyme is also dependent on this free phenolic group being present in an unionized form. Further experiments are now being conducted in these Laboratories in order to test the validity of these various possibilities.

SUMMARY

1. Human-liver arylsulphatase B has been considerably purified by a procedure involving acetone-fractionation, treatment with protamine sulphate and adsorption of the enzyme on sintered-glass disks.

2. During the early stages of the procedure the enzyme was markedly affected by buffer concentration and gave anomalous substrate concentration–activity curves. These effects were not obtained with the final enzyme preparation. Comparatively crude preparations of the corresponding ox-liver enzyme exhibited similar anomalies which, however, were not removed by further purification.

3. With dipotassium 2-hydroxy-5-nitrophenyl sulphate as assay substrate some indications of the nature of the ionizing groups which are involved in the formation and subsequent breakdown of the enzyme–substrate complex have been obtained from a study of the variation of K_m and $V_{max.}$ with pH.

4. The enzyme showed little activity towards potassium phenyl sulphate and its monosubstituted derivatives; there was appreciable activity towards disubstituted derivatives.

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Biological Synthesis of Ascorbic Acid: L-Galactono-γ-lactone Dehydrogenase

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The terminal step in the biosynthesis of L-ascorbic acid in peas has been shown to be the oxidation of L-galactono-γ-lactone by an enzyme present in mitochondria (Mapson, Isherwood & Chen, 1954). This enzyme has been solubilized from mitochondria obtained from freshly germinated peas, cabbage leaves and cauliflower florets. Since the enzyme preparations from the mitochondria of the cauliflower florets were found to be more active than those from either peas or cabbage, we have used this material in most of the work reported here. The present paper describes the preparation and partial purification of the enzyme, together with an account of some of its properties.

EXPERIMENTAL

Chemicals

L-Galactono-γ-lactone was prepared by the reduction of D-galacturonic acid by borohydride as follows. D-Galacturonic acid (10 g.) was dissolved in 40 ml. of water and neutralized with NaOH to between pH 8·5 and 9·0. Borohydride was added gradually with stirring at room temperature. Samples were removed and acidified with acetic acid to remove excess of borohydride, and galacturonic acid was tested for by boiling with Fehling's solution. After reduction was complete, the solution was acidified with acetic acid to pH 5·0, a slight excess of barium acetate added, and the precipitate filtered off. Ethanol (2 vol.) was added to the solution and the precipitate was collected. After the precipitate had been washed twice with 60% (v/v) ethanol, barium was removed by Dowex 50 resin, and to the filtrate 1–2 drops of 3N-HCl were added. The solution was concentrated to a syrup and dried *in vacuo*. The lactone was recrystallized from absolute ethanol.

L-Gulono-, D-altrono-, D-glucono- and D-mannono-γ-lactone were prepared as described by Isherwood, Chen & Mapson (1954). Glucurono-γ-lactone, D-gulono-γ-lactone and D-galactono-γ-lactone were purchased from L. Light and Co., Colnbrook, Bucks. Cytochrome *c* was prepared from ox heart by the method of Keilin & Hartree (1937).

Phenazine methosulphate was prepared by the addition of dimethyl sulphate to phenazine dissolved in nitrobenzene (Kehrmann, 1913; Hillemann, 1938). Flavin mononucleotide (FMN) and flavinadenine dinucleotide (FAD) were purchased from the Sigma Chemical Co., U.S.A.

Preparation of the enzyme

Extraction from mitochondria step 1. The florets (1 kg.) of cauliflower after separation from leaves and stalk were ground in a mechanical mortar with 1 l. of a sucrose–phosphate solution (0·4M-sucrose and 0·1M-Na phosphate buffer, pH 7·4). The mortar was cooled to −20° and the sucrose phosphate solution to 0° before grinding, which was carried out as rapidly as possible (usually within 30 min.) at room temperature. The mixture was pressed through muslin and centrifuged at 20 000 *g* for 20 min. The spun-down mitochondria were washed with 0·1M-phosphate buffer (pH 7·4), recentrifuged at 20 000 *g* for 20 min., and resuspended in 50–100 ml. of the same buffer. To this suspension was slowly added 10 times the volume of acetone, previously cooled to −20°. After 15 min. the precipitate was collected, the acetone partially removed *in vacuo*, and the residue suspended in 150 ml. of the phosphate buffer (0·1M). The suspension was dialysed against at least two changes of the same buffer for 3 hr. at +1° to remove most of the acetone.

Fractionation with ammonium sulphate step 2. The suspension was centrifuged to remove undissolved protein, and the clear solution brought to 40% saturation by the addition of 243 g. of $(NH_4)_2SO_4$/l. of solution. The precipitated protein was removed, and the solution brought to 70% saturation by the further addition of 205 g. of $(NH_4)_2SO_4$/l. The precipitate was collected, dissolved in 20 ml. of 0·01M-Na

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