Studies on Sulphatases

27. THE PARTIAL PURIFICATION AND PROPERTIES OF THE ARYLSULPHATASE OF THE DIGESTIVE GLAND OF *HELIX POMATIA*

By K. S. DODGSON AND GILLIAN M. POWELL

Department of Biochemistry, University of Wales, Newport Road, Cardiff

In the preceding paper (Dodgson & Powell, 1959) it was shown that an arylsulphatase present in the digestive juice and the digestive gland of the edible snail exhibited a shift in optimum pH as the concentration of substrate was increased. This paper describes the partial purification of the digestive-gland enzyme and provides further evidence that the shift in optimum pH is not due to the presence of more than one arylsulphatase.

MATERIALS AND METHODS

Substrates. Potassium *p*-nitrophenyl sulphate (NPS) and dipotassium 2-hydroxy-5-nitrophenyl sulphate (NCS) were prepared as described by Dodgson & Powell (1959); the monopotassium salts of 4-hydroxy-2-nitrophenyl sulphate and 4-hydroxy-3-nitrophenyl sulphate were prepared by the method of Smith (1951).

Determination of enzyme activity. Arylsulphatase activity towards NPS and NCS was measured by the methods previously described (Dodgson & Powell, 1959). When the monopotassium salts of 4-hydroxy-3-nitrophenyl sulphate and 4-hydroxy-2-nitrophenyl sulphate were used as substrates, the method of determining enzyme activity was identical with that used for NPS and NCS, except that the spectrophotometric measurement of the final colour was at 540 mμ. Except where otherwise stated, incubation of enzyme (0·2 ml.) and substrate (0·2 ml.) was for 15 min. in the presence of 0·25 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris), which had been adjusted to the required pH with acetic acid. Enzyme concentrations were adjusted to give final spectrophotometric readings (log I_0/I) of less than 1·0. For reasons which are outlined below, enzyme and substrate were always mixed by rotating the reaction tubes between the hands.

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

Determination of protein. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Ratio of enzyme activity at low and high pH. Dodgson & Powell (1959) for convenience used the terms low and high pH to describe the two sets of experimental conditions for measuring enzyme activity. In the present work low pH conditions for NCS were pH 6·6 and 0·5 mM, and for NPS pH 6·25 and 0·5 mM. The corresponding high pH conditions were pH 7·4 and 15 mM-NCS and pH 7·5 and 15 mM-NPS.

EXPERIMENTAL AND RESULTS

Purification procedure

Stage 1. An aqueous extract of the digestive glands of starved snails was made as described by Dodgson & Powell (1959). The clear extract was dialysed for 24 hr. against water (100 vol.) at 2°.

Stage 2. The extract was adjusted to pH 6·0 and treated at 0° with M-MnCl$_2$ (5 ml. for each 50 ml. of extract) as the same pH. After standing at 0° for 45 min. the copious precipitate was separated by centrifuging at 0°. The clear supernatant was adjusted to pH 8·0 with N-NaOH and, after standing at 0° for 20 min., the precipitate was removed by centrifuging. The supernatant was then dialysed overnight at 2° against water (50 vol.).

Stage 3. Acetone was added slowly (with gentle stirring) to the enzyme solution at −5° to give a final concentration of acetone of 47% (v/v). After standing at −5° for 30 min. the precipitate was separated by centrifuging at −5° and discarded. The concentration of acetone in the supernatant was then increased to 57% (v/v) and, after standing at −5° for 15 min., the precipitate was collected by centrifuging at the same temperature. The precipitate was dissolved in ice-cold water (50 ml. for each 50 ml. of the original extract) and dialysed overnight at 2° against water (50 vol.).

Stage 4. The enzyme solution was adjusted to pH 5·0 with a trace of acetic acid, and solid (NH$_4$)$_2$SO$_4$ was added over a period of 30 min., with gentle stirring until the solution was 45% saturated with respect to (NH$_4$)$_2$SO$_4$. Frequent checks on the pH of the solution were made during this addition and when necessary it was readjusted to pH 5·0 with a trace of N-NaOH. After standing at 0° for 2 hr. the precipitate was removed by centrifuging and the concentration of (NH$_4$)$_2$SO$_4$ in the supernatant was increased to 70% saturation. The whole was kept at 0° for 2 hr., when the precipitate was separated by centrifuging, dissolved in ice-cold water (30 ml./50 ml. of original extract) and dialysed overnight at 2° against water (50 vol.).

Stage 5. The dialysate was adjusted to pH 7·0 and fractionated between 55 and 70% saturation with (NH$_4$)$_2$SO$_4$. After standing for 2 hr. at 0°, the precipitate was separated, dissolved in ice-cold water (30 ml./50 ml. of original extract) and dialysed for 24 hr. against several changes of water at 2°. The final enzyme solution was stored in the frozen state in stoppered plastic containers.

Table 1 shows the enzyme activities towards NCS at stages 1, 2, 3 and 5 of a typical purification. One of the main aims of the procedure was to remove nucleic acid, in view of its known tendency to combine readily with protein and thereby to be a possible contributory factor to the

Table 1. *Arylsulphatase activity of the digestive gland of* Helix pomatia *at various stages of the purification procedure*

Enzyme activity was measured against dipotassium 2-hydroxy-5-nitrophenyl sulphate (NCS) in the presence of 0·25 M-acetic acid–tris buffer at low pH (pH 6·6 and 0·5 mM-NCS) and high pH (pH 7·4 and 15 mM-NCS) as described in the text. One unit of arylsulphatase activity is equivalent to 1 μg. of 4-nitrocatechol liberated/15 min. at 38°.

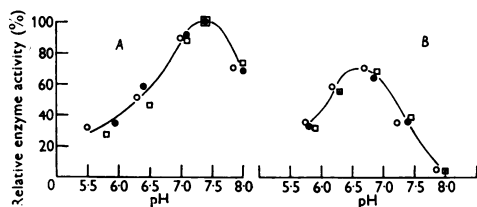| Purification stage	Enzyme activity (units/mg. of protein)		Ratio of activities at low and high pH	Concn. of nucleic acid (%)
	Low pH	High pH		
1	2 980	4 430	1:1·5	2·5
2	3 800	5 500	1:1·4	0
3	21 000	29 400	1:1·4	—
5	54 850	76 500	1:1·4	—

Fig. 1. Effect of pH on the arylsulphatase of *Helix pomatia* digestive gland at stages 1, 3 and 5 of the purification procedure. *A*, Measured against 15 mM-dipotassium 2-hydroxy-5-nitrophenyl sulphate (NCS) in the presence of 0·25 M-acetic acid–tris buffer; *B*, measured against 0·5 mM-NCS in the same buffer. ●, Stage 1; □, stage 3; ○, stage 5. For the purpose of clarity all the experimental points have been plotted as a percentage of the enzyme activity obtained at pH 7·4 and 15 mM-NCS.

Table 2. *Michaelis constants at pH 6·6 and 7·4 for the arylsulphatase of* Helix pomatia *digestive gland at stages 1, 3 and 5 of the purification procedure*

The enzyme was acting on dipotassium 2-hydroxy-5-nitrophenyl sulphate in the presence of 0·25 M-acetic acid–tris buffer at 38°.

Purification stage	$10^4 K_m$	
	pH 6·6	pH 7·4
1	1·4	1·7
3	1·3	4·7
5	0·9	7·3

shift in pH optimum. Treatment with $MnCl_2$ was effective in eliminating nucleic acid. On the other hand, attempted removal of nucleic acid with protamine (cf. Dodgson & Lloyd, 1957) resulted in substantial losses in enzyme activity.

Appreciable losses in enzyme activity occurred during purification, but these losses were considerably increased if the procedure was not strictly adhered to. The final preparation usually contained about 10% of the activity of the initial extract. A number of other procedures which were attempted, including the use of adsorbents, electrophoresis and fractionation on carboxymethylcellulose ion-exchange columns, resulted in large and often complete losses of enzyme activity.

Properties of the enzyme at various stages of purification

Table 1 shows that the ratio of enzyme activity at low and high pH did not vary significantly during purification. This finding provides further evidence for the existence of one arylsulphatase only in the enzyme preparation. In order to obtain additional support for this view the properties of the enzyme preparation at stages 1, 3 and 5 were compared.

Effect of pH and substrate concentrations. Fig. 1 shows the pH–activity curves for the three stages with NCS at low and high concentrations. The curves obtained for each stage were similar. Substrate concentration–activity curves towards NCS at pH 6·6 and 7·4 for the three stages provided no indication of the presence of more than one arylsulphatase. K_m values for each stage, calculated from the plot of v against $v/[S]$, are listed in Table 2. At high pH there was a steady decrease in the affinity of enzyme for substrate as the purity of the preparation increased, whereas, at low pH, the opposite effect was obtained.

Effect of time of incubation and enzyme concentration. Fig. 2 shows the effects of the time of incubation on the arylsulphatase activity of the three stages when measured at both low and high pH. In each case there was a marked decline in enzyme activity as the reaction proceeded. All three stages gave linear enzyme concentration–activity curves at both low and high pH. There was no evidence from the curves of the presence of an endogenous inhibitor or activator.

Effect of buffer concentration. The increase in enzyme activity with increasing concentration of buffer (either acetic acid–tris or acetic acid–sodium acetate) which had previously been observed for the crude extract of the digestive gland (Dodgson & Powell, 1959) was still obtained with stages 3 and 5. In each case the extent of the increase when measured at low pH paralleled that obtained at high pH, suggesting that the effect was due to a single enzyme.

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Instability of the enzyme

During the initial studies on the purification of the digestive-gland enzyme considerable difficulty was experienced in obtaining satisfactory duplicate determinations when measuring enzyme activity. The reason for this was ultimately traced to the fact that enzyme and substrate solutions were being mixed by agitation of the centrifuge tubes in which the determinations were made. Losses in enzyme activity were occurring as a result of this procedure. These losses were eliminated when substrate and enzyme were mixed by rotation of the reaction tubes between the hands. A similar effect has been noted by Morton (1957) with alkaline phosphatase.

A series of experiments showed that when the stage 1 enzyme and the substrate (both at 38°) were mixed by brief agitation, as opposed to rotation, up to 10% loss in enzyme activity occurred. With the stage 5 enzyme the corresponding figure approximated to 20%. Agitation of the warm enzyme solution before the addition of substrate resulted in only slight losses in enzyme activity. Other experiments were made in which the incubation mixtures were briefly agitated every 1 min. during the incubation period (15 min.). With the stage 1 enzyme up to 30% of the activity was lost as a result of this treatment, whereas with the stage 5 enzyme between 60 and 70% of the activity disappeared. The losses in activity were not due to adsorption of the enzyme on glass surfaces [cf. acid phosphatase (Jeffree, 1957) and testicular hyaluronidase (Rasmussen, 1954)] since similar losses were obtained with plastic tubes.

Other observations indicated that the stage 5 enzyme was particularly unstable. Thus appreciable losses in enzyme activity occurred during any procedure where frothing of the enzyme solution occurred. Freezing and thawing also resulted in destruction of the enzyme, as did storage of the enzyme (particularly diluted enzyme) in the liquid state. It is perhaps significant that, in all cases where loss of enzyme activity was experienced, the percentage decrease in activity as measured at low pH approximately paralleled that measured at high pH.

Further observations on the pH shift

Action of the enzyme on other substrates. A pH shift similar to that observed for the stage 5 enzyme acting on NCS was also obtained with other arylsulphatase substrates. With NPS in the presence of 0·25 M-acetic acid–tris buffer the shift ranged from pH 6·25 at a substrate concentration of 0·5 mM to pH 7·5 at 15 mM. Table 3 records the relative enzyme activities and Michaelis constant (K_m, calc. from the plot of v against $v/[S]$) for NPS and NCS at the lower and upper limits of the pH shift. A comparison of the values for NCS and NPS at low pH together with a comparison of the corresponding values at high pH shows that the enzyme exhibits relatively greater affinity and activity towards NCS.
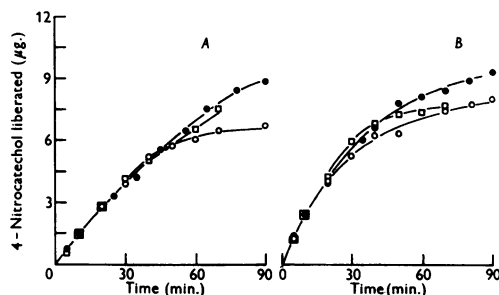

Fig. 2. Effect of time of incubation on the arylsulphatase of *Helix pomatia* digestive gland at stages 1, 3 and 5 of the purification procedure. *A*, Measured against 0·5 mM-dipotassium 2-hydroxy-5-nitrophenyl sulphate (NCS) in the presence of 0·25 M-acetic acid–tris buffer, pH 6·6; *B*, measured against 15 mM-NCS in the same buffer, pH 7·4. ●, Stage 1; □, stage 3; ○, stage 5. Stage 3 and 5 enzyme concentrations were adjusted so that the amount of 4-nitrocatechol liberated in 10 min. was equal to that liberated by the stage 1 enzyme in the same period.

Table 3. *Relative enzyme activities and Michaelis constants (K_m) for the arylsulphatase of* Helix pomatia *digestive gland at stage 5 of the purification procedure*

The enzyme was acting on dipotassium 2-hydroxy-5-nitrophenyl sulphate (NCS) and potassium *p*-nitrophenyl sulphate (NPS) at the lower and upper limits of the pH shift in the presence of 0·25 M-acetic acid–tris buffer. Arylsulphatase activity is expressed in terms of μg. of 4-nitrocatechol or *p*-nitrophenol liberated/mg. of protein/15 min. at 38°.

Substrate	Concn. of substrate (mM)	pH	Arylsulphatase activity	$10^4 K_m$
NPS	0·5	6·25	18 800	2·7
	15·0	7·5	49 700	51·0
NCS	0·5	6·6	54 850	0·9
	15·0	7·4	76 500	7·3

Fig. 3 shows the effect of increasing substrate concentration on the optimum pH of the stage 5 enzyme acting on the monopotassium salts of 4-hydroxy-2-nitrophenyl sulphate and 4-hydroxy-3-nitrophenyl sulphate in the presence of 0·25 M-acetic acid–sodium acetate buffer. In each case there was a shift in pH optimum but the extent of the shift was less marked than that obtained with NPS or NCS.

Treatment with disodium ethylenediaminetetra-acetate. Mention has already been made (Dodgson & Powell, 1959) that treatment of the crude enzyme extract of the digestive gland with sodium ethylenediaminetetra-acetate (EDTA) had no effect on the shift in pH optimum. This suggested that the presence of a metal activator or inhibitor could not be responsible for the phenomenon (cf. alkaline phosphatase, Morton, 1957). Further attempts were made to remove possible trace metals from the stage 5 enzyme with a procedure similar to that described by Morton (1957).

The enzyme solution (1 ml.) was mixed with 4 ml. of 0·2 M-EDTA which had been adjusted to pH 8·0 with ethanolamine. The solution was then dialysed for 24 hr. at at 2° against 1 l. of 0·1 M-EDTA which had been adjusted to the same pH, and subsequently for 48 hr. against two changes of water (2 l. in all). The activity of the final dialysate at low and high pH was compared with that of the original enzyme preparation and with enzyme which had been subjected to the dialysis procedures except that water was substituted for EDTA. As a further control, 1 ml. of enzyme was diluted with 4 ml. of water and allowed to stand at 2° for the same period of time. All enzyme solutions were adjusted to the same volume before assay.

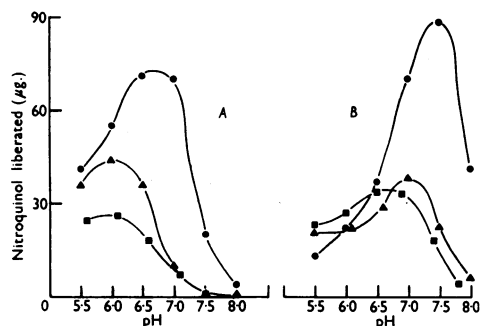No loss in enzyme activity occurred as a result of the treatment with EDTA. On the other hand,

some loss in enzyme activity was observed with the enzyme preparation which had been dialysed against water only (17 % loss) and with the preparation which had been allowed to stand at 2° (6 % loss). The presence of an endogenous metal activator or inhibitor in the stage 5 enzyme therefore seems unlikely. Mg^{2+}, Mn^{2+} and Zn^{2+} ions had no activating effect on the enzyme (see Table 4).

Possible inhibitory effects of hydroxyl ions. The shift in pH optimum could possibly be explained if OH^- ions were acting as competitive inhibitors of the enzyme. Such inhibition would be greatest at low substrate concentrations and would result in an apparent shift in the pH optimum of the enzyme in the direction of lower pH as the substrate concentration was decreased. To explore this possibility a study was made of the effect of substrate (NCS) concentration on the activity of three different concentrations of the enzyme at pH 7·4 in the presence of 0·25 M-acetic acid–tris buffer. Under these circumstances the concentration of OH^- ions remained constant but the concentration of the enzyme and hence the enzyme–inhibitor (OH^- ions) ratio was varied. Fig. 4 shows the substrate concentration–activity curves obtained. K_m values (calc. from the plot of v against $v/[S]$) for relative enzyme concentrations of 1, 2 and 3 were 0·52, 0·73 and 0·64 mM respectively. These values can probably be considered as identical within the limits of experimental error and provide no indication of inhibition by OH^- ions.

Fig. 3. Effect of substrate concentration on the pH optimum of the arylsulphatase of *Helix pomatia* digestive gland (stage 5) acting on: *A*, monopotassium 4-hydroxy-3-nitrophenyl sulphate in the presence of 0·25 M-acetic acid–sodium acetate buffer; *B*, monopotassium 4-hydroxy-2-nitrophenyl sulphate in the same buffer. ■, 0·5 mM; ▲, 15 mM; ●, 15 mM. The concentration of enzyme used to obtain the curves in *A* was five times that used to obtain the curves in *B*.
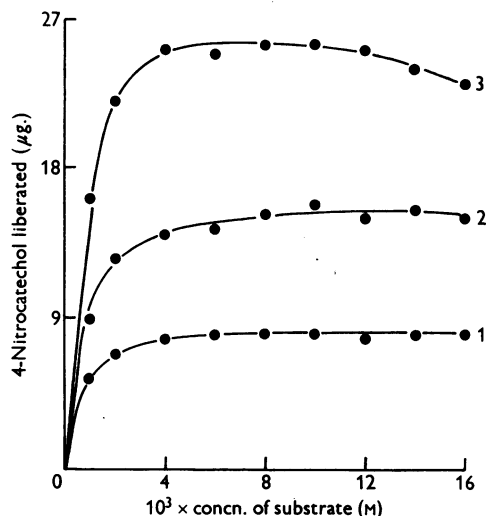

Fig. 4. Effect of the concentration of dipotassium 2-hydroxy-5-nitrophenyl sulphate on three different concentrations of the stage 5 enzyme of *Helix pomatia* digestive gland acting in the presence of 0·25 M-acetic acid–tris buffer, pH 7·4. The curves are numbered in terms of the relative enzyme concentration used.

Effect of certain compounds on enzyme activity at low and high pH. A study was made of the effects of possible inhibitors and activators on enzyme activity towards NPS and NCS at both low and high pH. In these experiments a slight modification of the method of measuring enzyme activity was made since some of the compounds tested (e.g. Na_2SO_3, $NaHSO_3$, phenylhydrazine and hydroxylamine) are capable, under certain conditions, of hydrolysing one or both of the assay substrates (Dodgson & Powell, unpublished results).

Enzyme (0·2 ml.) and inhibitor (0·2 ml. of a 0·1 M-solution in 0·5 M-acetic acid–tris buffer at the appropriate pH) were pre-incubated for 3 min. at 38° before adding 0·4 ml. of a solution of the substrate in 0·25 M-acetic acid–tris buffer at the same pH. Suitable controls were made in which 0·5 M-acetic acid–tris buffer was substituted for buffered inhibitor and, as a control of possible non-enzymic hydrolysis of substrate by inhibitor, further determinations were made in which inhibitor and boiled enzyme were incubated with substrate.

Table 4 shows that phosphate, sulphite, bisulphite and fluoride were the most powerful inhibitors of *Helix* arylsulphatase. In contrast, cyanide had little effect on enzyme activity. The results provide no clear indication whether a single enzyme only is involved, although, in general, where inhibition occurs the effects are seen at both low and high pH. It will be observed that phosphate, fluoride, sulphite and sulphate exert greatest inhibitory effects at low pH values. On the other hand, semicarbazide, hydroxylamine and (towards NCS) phenylhydrazine exert their greatest effects at high pH values. This may at first suggest
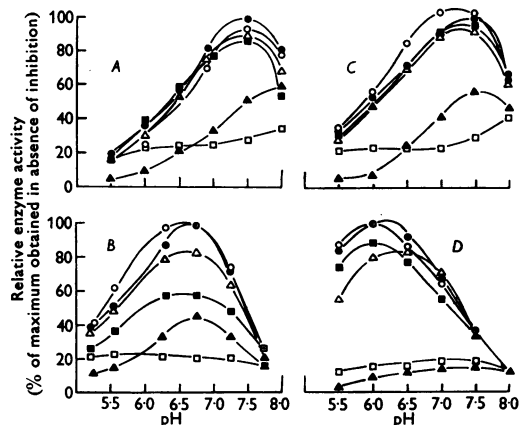

Fig. 5. Effect of certain compounds (at a concentration of 25 mM) on the pH optimum of the arylsulphatase of *Helix pomatia* digestive gland (stage 5) acting on dipotassium 2-hydroxy-5-nitrophenyl sulphate (NCS) and potassium *p*-nitrophenyl sulphate (NPS) in the presence of 0·25 M-acetic acid–tris buffer. *A*, 15 mM-NCS; *B*, 0·5 M-NCS; *C*, 15 mM-NPS; *D*, 0·5 mM-NPS. ●, Control; ○, NaCl; ■, NaCN; □, Na_2HPO_4–NaH_2PO_4; ▲, NaF; △, Na_2SO_4.

Table 4. *Effects of certain compounds on the activity of the arylsulphatase of the digestive gland of* Helix pomatia *acting on potassium* p-*nitrophenyl sulphate and dipotassium 2-hydroxy-5-nitrophenyl sulphate at low and high* pH

Results are expressed as a percentage of the enzyme activity obtained in the absence of inhibitor or activator. The terms low and high pH are explained in the text. Except where indicated, the final concentration of inhibitor or activator was 25 mM.

| | Enzyme activity (%) | | | |
| | NPS | | NCS | |
Compound added	Low pH	High pH	Low pH	High pH
Na_2SO_3	4	5	1	5
$NaHSO_3$	1	6	1	6
NaF	35	47	11	55
Na_3PO_4	20	24	18	26
$Na_4P_2O_7$	48	95	29	86
Na_2SO_4	61	86	89	95
NaCN	88	91	94	96
NaCl	100	94	100	105
Sodium citrate	92	80	94	87
Sodium benzene sulphonate	90	95	64	92
Potassium ethyl sulphate	100	86	86	102
Urea	100	100	97	95
Hydroxylamine	65	34	89	51
Semicarbazide	102	91	98	83
Hydrazine	101	103	100	89
Phenylhydrazine	77	61	67	83
$MgCl_2$*	83	85	90	89
$MnCl_2$*	80	81	89	84
$ZnCl_2$*	64	77	74	84

* Final concn. 1 mM.

that two enzymes are present in the enzyme preparation. However, it is more probable that the contrast merely reflects the difference between anionic inhibitors, on the one hand, and those which can be regarded as cationic, on the other.

Fig. 5 shows the effects on the pH curves at low and high concentrations of substrate (NCS and NPS) of some of the compounds which were tested as inhibitors. With the exception of fluoride and phosphate, which tended (particularly when measured at high substrate concentrations) to shift the pH optima in the direction of higher pH, the compounds had little effect on the pH optima. A similar effect of fluoride and phosphate had previously been noted for the type 2 arylsulphatase of *Proteus vulgaris* (Dodgson, 1959).

DISCUSSION

The collective results provide some indication that the shift in pH optimum with increasing substrate concentration cannot be attributed to the presence of more than one arylsulphatase in the enzyme preparation. Mention has already been made that a number of other hydrolytic enzymes exhibit a similar phenomenon (alkaline phosphatase, Morton, 1957; β-glucosidase, Hofstee, 1955; β-glucuronidase, Cox, 1959), and a number of possible explanations of this effect can be advanced. The theoretical background to these explanations has been dealt with by Bull (1954) and by Friedenwald & Maengwyn-Davies (1954) and will not therefore be re-iterated here. It is sufficient to say that the effect might be observed with any enzyme-substrate interaction in which several equilibria are involved or where 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. Situations in which the overall reaction involves a number of different equilibria could result, for example, from the participation of an activator (e.g. a metal, H^+ or OH^- ions) in the catalytic process. An explanation based on these lines has been advanced by Morton (1957) to account for the variation with substrate concentration of the optimum pH of alkaline phosphatase, an enzyme whose activity depends on the presence of a metal (usually Mg^{2+} ions). Morton has pointed out that, for the overall enzymic reaction, ten different equilibria must be considered. Three of these can be regarded as competitive routes for the formation of the metal-enzyme-substrate complex and a fourth shows that the effective concentration of metal activator will be influenced by the concentration of OH^- ions and hence pH. Motzok & Branion (1959), working with chick alkaline phosphatase, also

suggest that the inhibitory effect of OH^- ions might explain the shift in pH optimum exhibited by this enzyme. However, similar considerations do not apply to *Helix* arylsulphatase since OH^- ions appear to have no inhibitory effect on the enzyme, the activity of which appears to be independent of the presence of a metal activator. Mention has previously been made that the enzyme concentration-activity curves for *Helix* arylsulphatase provide no indication of the presence of endogenous inhibitors or activators.

Another type of situation where several equilibria must be considered will arise when the substrate is capable of existing in more than one ionic form (Friedenwald & Maengwyn-Davies, 1954). Cox (1959) has suggested that the shift in pH optimum with change in concentration of substrate which occurs when molluscan β-glucuronidase is acting on phenolphthalein glucuronide can be explained in this way. Cox suggests that the enzymic reaction requires the participation of un-ionized substrate. At low substrate concentrations the concentration of un-ionized phenolphthalein glucuronide (pK of the substrate carboxyl group is about 3·0) might be the limiting factor and decrease in pH towards 3·0 would increase the un-ionized substrate concentration. It follows that the lower the substrate concentration, the lower would be the apparent optimum pH. In *Helix* arylsulphatase the free phenolic hydroxyl group present in one of the substrates used, namely NCS, is known to have pK about 6·5 (Dodgson, Spencer & Williams, 1955). Although no direct check has been made similar considerations will probably apply to the free phenolic hydroxyl groups of two of the other substrates used, namely potassium 4-hydroxy-3-nitrophenyl sulphate and potassium 4-hydroxy-2-nitrophenyl sulphate. With all three substrates, over the pH range studied, the proportions of ionized (with respect to the phenolic hydroxyl group) to un-ionized substrate will vary with pH. However, this apparently in no way contributes to the observed shift in pH optimum since the shift is also obtained with NPS, a substrate which does not possess a free phenolic hydroxyl group.

Hofstee (1955) has shown that the pH optimum of β-glucosidase shifts from about 3·5 at concentrations of substrate (salicylic acid β-glucoside) below 1 mM to about 4·5 at a substrate concentration of 33 mM. Hofstee attributes this shift to the difference in the influence of pH on K_m and $V_{max.}$, i.e. on the formation and the breakdown of the enzyme-substrate complex respectively. This may be the explanation of the pH shift with *Helix* arylsulphatase, although there is, as yet, no evidence to support this possibility. However with human arylsulphatase B (Dodgson & Wynn, 1958) and ox arylsulphatase B (Webb & Morrow, 1959),

both of which exhibit a number of properties in common with the *Helix* enzyme, the formation of the enzyme–substrate complex and the subsequent breakdown thereof can be influenced independently by variation of experimental conditions. No attempt has yet been made to test this possibility for the *Helix* enzyme. Buffer concentration has a marked effect on the activity of this enzyme and this effect probably reflects the comparative impurity of the enzyme preparation. Thus a similar effect has been observed by Dodgson & Wynn (1958) for comparatively crude preparations of both human and ox arylsulphatase B, but the effect is no longer obtained with the highly purified enzymes (Dodgson & Wynn, 1958; Webb & Morrow, 1959). It therefore seems desirable to obtain *Helix* arylsulphatase in a highly purified form before pursuing further the problem of the shift in pH optimum. As a preliminary to this further purification a study is now being made of possible ways of protecting the enzyme from denaturation during the purification procedure.

Dodgson & Spencer (1957) have suggested that the arylsulphatase enzymes appear to fall into two groups. The type I arylsulphatases have a high affinity for, and are particularly active towards, simple arylsulphates such as NPS. They are not markedly affected by phosphate and fluoride but are strongly inhibited by cyanide. In contrast the type II arylsulphatases show greatest affinity and activity towards more complex arylsulphates such as NCS and are strongly inhibited by phosphate and fluoride but not by cyanide. On the basis of these suggestions *Helix* arylsulphatase would be classified as a type II enzyme. Thus the enzyme shows a greater affinity and activity towards NCS than towards NPS (Table 3) and, although largely unaffected by cyanide, is inhibited strongly by phosphate and fluoride (Table 4). However, in certain respects the enzyme is unlike any other type II arylsulphatase previously studied. Thus the pH shift which occurs with increasing substrate concentration does not occur with the two other type II arylsulphatases (from *Patella vulgaris* and *Proteus vulgaris*) which have so far been investigated (Dodgson & Powell, unpublished results; Dodgson, 1959). Moreover, at least three of the type II arylsulphatases (human and ox arylsulphatases B and the enzyme of *Proteus vulgaris*) are known to be activated strongly (several-fold) by chloride when acting on NPS (Dodgson & Wynn, unpublished results; Webb & Morrow, 1959; Dodgson, 1959). This effect is not shown by the *Helix* enzyme (Fig. 5). Further work is continuing in these Laboratories to establish whether the arylsulphatases can be justifiably classified into two distinct types or whether there exists a whole 'spectrum' of enzymes between the two extremes.

Finally, with β-glucosidase, alkaline phosphatase and β-glucuronidase, all of which exhibit shifts in pH optima with increase in substrate concentration, the enzymes are able to catalyse the transfer of glucose, phosphate and glucuronic acid respectively to acceptors other than water (see, for example, Rabaté, 1935; Morton, 1958; Fishman & Green, 1957). The possibility that *Helix* arylsulphatase is capable of transferring sulphate from arylsulphates to acceptors other than water is now being examined.

SUMMARY

1. The arylsulphatase of the digestive gland of *Helix pomatia* has been partially purified by treatment with manganese chloride followed by fractionation with acetone and ammonium sulphate.

2. The enzyme became progressively more unstable as the purity increased but no other significant changes in the properties of the enzyme occurred during the purification procedure, and the final preparation was still exhibiting a shift in pH optimum in the direction of higher pH as the substrate concentration was increased.

3. Evidence suggests that the observed shift in pH optimum cannot be attributed to the presence of more than one arylsulphatase in the enzyme preparation.

4. Some arguments which have been used to explain similar shifts in pH optima which occur with other hydrolytic enzymes (e.g. alkaline phosphatase) have been shown to be inapplicable to the *Helix* enzyme.

5. The relative behaviour of the enzyme towards potassium *p*-nitrophenyl sulphate and dipotassium 2-hydroxy-5-nitrophenyl sulphate together with the effects of certain inhibitors suggests that the enzyme can be classified as a type II arylsulphatase.

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The Biosynthesis of Porphyrins from Porphobilinogen by *Rhodopseudomonas spheroides*

2. THE PARTIAL PURIFICATION AND SOME PROPERTIES OF PORPHOBILINOGEN DEAMINASE AND UROPORPHYRINOGEN DECARBOXYLASE*

By D. S. HOARE† AND H. HEATH

Department of Chemical Pathology, University College Hospital Medical School, London, W.C. 1

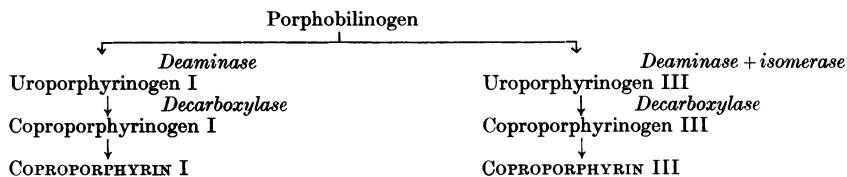It has been already established that preparations from the photosynthetic bacterium *Rhodopseudomonas spheroides* synthesize coproporphyrin III from porphobilinogen (Heath & Hoare, 1959*a, b*). It was also shown that under certain conditions porphobilinogen was converted into uroporphyrin I and uroporphyrin III and that uroporphyrinogens I and III were decarboxylated to yield, ultimately, corpoporphyrins I and III respectively (Hoare & Heath, 1958).

The recent work of Bogorad (1958*a, b, c*) with enzyme preparations from spinach, wheat germ and *Chlorella*, and that of Granick & Mauzerall (1958) and Mauzerall & Granick (1958) with enzyme preparations from chick and rabbit reticulocytes, has shown that at least three enzymes are involved in the conversion of porphobilinogen into coproporphyrin III. Porphobilinogen deaminase purified from spinach extracts (Bogorad, 1958*a*) converts porphobilinogen, via unknown intermediates, into uroporphyrinogen I. In a preliminary report Lockwood & Rimington (1957) described the partial purification of an enzyme from chick erythrocytes

which converted porphobilinogen into uroporphyrin III: the enzyme was called porphobilinogenase. When porphobilinogenase was preheated to 55° for 15 min. it catalysed the formation of uroporphyrin I from porphobilinogen. Similar effects of preheating had been observed earlier with crude preparations of *Chlorella* (Bogorad & Granick, 1953) and erythrocyte systems (Booij & Rimington, 1957). Similar investigations were reported in more detail by Granick & Mauzerall (1958), who purified a porphobilinogenase from chick erythrocytes and from rabbit reticulocytes. They established that uroporphyrinogen III was the reaction product in unheated preparations and uroporphyrinogen I was formed when preheated preparations were incubated with porphobilinogen. Uroporphyrinogen isomerase isolated from extracts of wheat germ (Bogorad, 1958*b*) catalysed the formation of uroporphyrinogen III from porphobilinogen in the presence of porphobilinogen deaminase; it had no action on uroporphyrinogen I or on porphobilinogen alone. Crude preparations of *Chlorella* were found to catalyse the formation of coproporphyrin from uroporphyrinogen (Bogorad, 1958*c*). Mauzerall & Granick (1958) partially purified a uroporphyrinogen decarboxylase from chick and rabbit

Porphobilinogen

↓ *Deaminase*	↓ *Deaminase + isomerase*
Uroporphyrinogen I	Uroporphyrinogen III
↓ *Decarboxylase*	↓ *Decarboxylase*
Coproporphyrinogen I	Coproporphyrinogen III
↓	↓
Coproporphyrin I	Coproporphyrin III

Scheme 1