The Interaction of some Factors Affecting the Kinetics of a Molluscan β -Glucuronidase

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Marine molluscs have been reported as convenient and rich sources of β -glucuronidase (Stitch & Halkerston, 1953; Dodgson & Spencer, 1953; Cox, 1957). These β -glucuronidase preparations have been useful for the hydrolysis of urinary steroid glucuronides. In the application of enzyme preparations for such hydrolyses, rapid reactions consistent with optimum yield of the liberated steroids are desired. In determining optimum conditions for hydrolysis, the effects of the factors pH, duration of reaction and enzyme concentration on the rate of hydrolysis are generally studied with one factor at a time being varied; the interactions of factors affecting the hydrolyses have been neglected. Such interactions are known to affect chemical reactions and may be investigated by factorial tests, in which all possible combinations of all chosen levels of each factor under test are investigated, either together or in blocks or fractional replicates. The use of factorial tests and the general desirability of this form of experimentation has been discussed thoroughly by Fisher (1949) and by Cochran & Cox (1957).

The results presented here give some properties of a molluscan β -glucuronidase prepared from the limpet *Cellana tramoserica* (Cox, 1957). Before a study of the optimum conditions for enzymic hydrolysis of steroid conjugates, the hydrolysis of phenolphthalein glucuronide has been studied to determine whether interactions occur between the factors which affect the enzymic hydrolysis. Highly significant interactions have been found.

MATERIALS AND METHODS

Reagents. Acetone A.R. was fractionally distilled before use. Glycine, reagent grade, was obtained from British Drug Houses Ltd. and sodium phenolphthalein glucuronide from L. Light and Co. Trichloroacetic acid, acetic acid and sodium hydroxide were A.R. grade.

β -Glucuronidase. This was prepared from the limpet *Cellana tramoserica* (Cox, 1957) by the procedure of Dodgson & Spencer (1953), in which the visceral humps are homogenized in chilled acetone. Preparations at two stages of purification have been used. The product after the first acetone precipitation (A) contained 2×10^5 β -glucuronidase units/g. This preparation is suitable for use in the enzymic hydrolysis of steroid glucuronides. The enzyme was further

purified by reprecipitating the water-soluble fraction with 6 vol. of acetone at 0°, yielding an off-white water-soluble powder (B) with an activity of 3×10^5 β -glucuronidase units/g. These two products correspond to powders A and B respectively of Dodgson & Spencer (1953). Solutions of β -glucuronidase were prepared by grinding 100 mg. of acetone-dried enzyme with 1–2 ml. of water to obtain a paste. The paste was diluted to 20 ml. and filtered through a cotton-wool plug. Dilutions of this concentrated enzyme solution were prepared as required. Fresh solutions were prepared from the enzyme powder for each experiment.

pH Measurements. A glass electrode and a Radiometer model 22 pH meter (Radiometer, Copenhagen) were used.

Activity of β -glucuronidase preparations. This was determined by the method of Bernfeld & Fishman (1953). One unit of enzyme liberates 1 μ g. of phenolphthalein/hr. from an mM-solution of phenolphthalein glucuronide at pH 4.3 in 0.1M-acetic acid-sodium acetate buffer at a temperature of 37°.

General procedure for hydrolyses. The hydrolysis of phenolphthalein glucuronide under various conditions was followed by measuring the amount of phenolphthalein liberated (Talalay, Fishman & Huggins, 1946; Bernfeld & Fishman, 1953). Substrate and enzyme solutions were adjusted to the required pH with M-acetic acid before use. Mixtures of 0.10 ml. of substrate solution, 0.40 ml. of 0.1M-acetic acid-sodium acetate buffer of the required pH and 0.50 ml. of enzyme solution at 37° were incubated in a water bath at $37^\circ \pm 0.1$ for the required time, generally 1 hr. The reactions were stopped by adding 1.0 ml. of 5% (w/v) trichloroacetic acid solution. After the addition of 2.5 ml. of glycine buffer (Bernfeld & Fishman, 1953) and 1.5 ml. of water the extinction coefficients of the solutions at 552 m μ were measured. Blank estimations were made by incubating the substrate-buffer mixture and enzyme separately for the required time, and adding 1.0 ml. of trichloroacetic acid solution to the latter followed by substrate-buffer mixture. The rest of the procedure was as used for estimations. Blank estimations generally had an extinction coefficient of zero within reading error when compared with water. The final solutions for colorimetry should be pH 10.2. In preparing the glycine buffer the pH of a reaction blank was checked and, if necessary, the glycine buffer adjusted to ensure this value for the final pH of the colorimetric solutions.

Calibration curves relating the extinction coefficient of solutions to concentration of phenolphthalein were obtained by treating 1.0 ml. of aqueous solutions containing 0–60 μ g. of phenolphthalein with 1.0 ml. of 5% trichloroacetic acid, 2.5 ml. of glycine buffer and 1.5 ml. of water and determining the extinction coefficient of the mixture at 552 m μ .

RESULTS

Usual experimental method. The effect of pH, substrate concentration, enzyme concentration, duration and temperature of reaction on the rate of hydrolysis of phenolphthalein glucuronide were first investigated in the usual way, varying each factor separately.

With a substrate concentration of mM an optimum for the hydrolysis was at pH 4.2–4.4 (Fig. 1). The effect of substrate concentration on the reaction rate is shown in Fig. 2. Under the conditions used, in particular at pH 4.25, a value for K_m of 0.06 mM was obtained; this value was determined graphically from the plot of substrate concentration/velocity against substrate concentration. The amount of substrate hydrolysed was proportional to enzyme concentration (0.1–0.8 mg./ml.; s.d. of triplicates, 0.92 μ g. of phenolphthalein) and to reaction time (0.5–4.0 hr.; s.d. of quadruplicates, 1.5 μ g. of phenolphthalein) over the ranges tested. Apart from the variation in enzyme concentration or duration of hydrolysis in these two experiments, the standard conditions used were:

Fig. 1. Effect of pH on the reaction velocity for the hydrolysis of phenolphthalein glucuronide. Substrate concentration mM, hydrolysis for 1 hr. at 37°. Expt. 1, enzyme concentration 0.2 mg./ml. (○); Expt. 2, enzyme concentration 0.16 mg./ml. (●). Each point is the mean of two or three estimations, standard deviation for replicates 0.4 μ g. of phenolphthalein.

substrate concentration, mM; pH 4.25; temperature, 37°; enzyme concentration, 0.2 mg./ml.; duration of hydrolysis, 1 hr. Variation of the temperature at which the hydrolysis was carried out led to an increase in reaction velocity up to about 60°. Thereafter rapid heat-denaturation of the enzyme is evident (Fig. 3).

The factors tested in the above experiments produced effects which are commonly observed for an enzymic reaction. This normality is important in considering the marked interactions which were found between factors in the experiments described below.

Factorial experiment. The effect of the above factors was investigated in an experiment in which

Fig. 2. Variation of reaction velocity with substrate concentration. pH 4.25, enzyme concentration 0.2 mg./ml. and hydrolysis for 1 hr. at 37°. Plot of substrate concentration (s) against velocity (v), ○; plot of s/v against s , ●. Each point is the mean of three estimations, standard deviation 0.4 μ g. of phenolphthalein.

Fig. 3. Amount of phenolphthalein liberated with varying temperature. Substrate concentration mM, pH 4.25, enzyme concentration 0.1 mg./ml. and duration of hydrolysis 1 hr. Standard deviation 0.8 μ g. of phenolphthalein for quadruplicates.

all combinations of each factor and level of factor chosen were used. The results of such an experiment may be subjected to an analysis of variance to determine the interactions between factors.

The test was carried out as a 4×2^4 factorial with each of the factors (substrate concentration, enzyme concentration, duration of reaction and temperature) at two levels; four levels of pH were investigated (Table 1). The whole experiment was carried out simultaneously with one batch of reagents. The levels chosen for these factors were within the ranges in which effects of the factors were observed as described in the previous section.

The pH optimum curve shows no significant double curvature ($0.3 > P > 0.2$) determined from the results of the experiments shown in Fig. 1, i.e. there is no significant cubic component in the regression equation of 'phenolphthalein liberated' on 'pH'. Hence the double-curvature component of pH and the interactions involving it can be used as a measure of error, so avoiding the need to include duplicates in the factorial experiment. As a further check the variance of these components was shown by Bartlett's test to be homogeneous ($\chi^2_{(15)} = 7.36$; $0.95 > P > 0.90$) and not to differ significantly from the variance between duplicates

Table 1. *Summary of analysis of variance: rate of hydrolysis of phenolphthalein glucuronide by β -glucuronidase*

Factor		Levels tested		
Temperature (F)		37°, 57°		
Time (E)		1 hr., 2 hr.		
Enzyme concentration (D)		50, 100 μ g./ml.		
pH (P)		3.65, 4.25, 4.85, 5.45		
Substrate concentration (A)		1.0, 0.1 mM		
	Source of variation	Degrees of freedom	Variance ratio	Significance level
Main effects	F	1	1500	***
	E	1	860	***
	D	1	880	***
	P	2	62	***
	A	1	910	***
First-order interactions	EF	1	120	***
	DF	1	142	***
	FP	2	9.5	**
	FA	1	310	***
	ED	1	51	***
	EP	2	4.9	*
	EA	1	160	***
	DP	2	8.0	**
	DA	1	120	***
	PA	2	43	***
Second-order interactions	DEF	1	0.7	—
	PEF	2	0.6	—
	AEF	1	65	***
	PDF	2	2.2	—
	ADF	1	56	***
	APF	2	9.8	**
	PDE	2	1.9	—
	ADE	1	21	***
	APE	2	2.6	—
	APD	2	6.9	**
Third- and fourth-order interactions	PDEF	2	2.0	—
	ADEF	1	8.4	*
	APEF	2	0.8	—
	APDF	2	3.3	—
	APDE	2	1.7	—
Error	APDEF	2	0.8	—
	Cubic components of P and interactions involving it	16	Variance 1.99	

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

Sources of variation involving P are shown with 2 degrees of freedom only as the degree of freedom associated with the cubic components of P and interactions involving it are used as error.

in the other experiments carried out with the enzyme (Table 2).

The amounts of phenolphthalein liberated under the conditions used are plotted relative to the pH (Fig. 4). Since only four pH values were tested, only major changes in pH optima are readily apparent; the most marked changes in pH optima are associated with change in substrate concentration. An analysis of variance (Table 1) shows, in addition to the expected significant main-order effects, that all of the first-order and half of the

second-order interactions are significant, many at levels where $P < 0.001$. Thus, for example, the significant second-order interaction of substrate concentration-pH-temperature ($P < 0.01$) indicates that, within the ranges tested, variation of temperature, substrate concentration or pH affects the interrelation of the other two factors on the hydrolysis rate. For this enzymic reaction and under the conditions used it is important that optimum conditions for the reaction be determined factorially in view of these large interactions.

Further tests. It was decided to investigate in more detail the interaction between substrate concentration and pH. This interaction may be of particular importance in obtaining optimum enzymic hydrolysis of steroid glucuronides, especially as such substrates in urine are commonly present in μM and lower concentrations. It is convenient to consider the interaction in terms of the effect of substrate concentration on the pH optimum. There is a marked shift in the pH optimum in this reaction; at the higher substrate concentrations (mM) an optimum near pH 4.8 was observed whereas at the lower substrate concentration (0.1 mM) no optimum was found within the range tested, although an optimum at a pH lower than 3.6 might be expected.

Table 2. *Bartlett's test for homogeneity of variances*

Source of variation	Degrees of freedom	Variance
Between quadruplicates (Fig. 3; reaction-time and enzyme-concentration experiments)	24	0.747
Between triplicates (Figs. 1 and 2)	30	0.920
Double-curvature components of pH and interactions involving it in factorial experiment (Table 1)	16	1.99
$\chi^2_{(2)} = 5.39.$		$0.95 > P > 0.90.$

Fig. 4. Results of factorial experiment plotted as effect of pH on reaction velocity. Substrate concentration 0.1 mM for sets A and B, and mM for sets C and D. Final enzyme concentration 0.1 mg./ml. for sets A and C, 0.05 mg./ml. for sets B and D. Levels of temperature are indicated as *F* (57°), *f* (37°) and duration of hydrolysis as *E* (2 hr.), *e* (1 hr.). Analysis of variance is given in Table 1.

Fig. 5. Effect of phenolphthalein glucuronide concentration on the pH optimum of β -glucuronidase. Substrate concentrations were as indicated, temperature 37° and hydrolysis for 1 hr. A, Enzyme preparation (A) at concentration of 0.17 mg./ml. B, Enzyme preparation (B) at concentration of 0.08 mg./ml. Standard deviation of replicates 0.4 $\mu\text{g.}$ of phenolphthalein.

Further experiments over a wider range of pH values confirmed the shift of the pH optimum with decrease in substrate concentration. The effect of pH on the liberation of phenolphthalein with substrate concentrations of from 1.0 to 0.05 mM is shown in Fig. 5. Two separate enzyme preparations were used, and also the first (A) was at the usual stage of purification, whereas the other (B) was taken past the second acetone precipitation (see Materials and Methods). Both preparations showed a shift in the pH optimum with change in substrate concentration. Over the pH ranges used (Figs. 4, 5) the enzyme can only be considered to be saturated at the highest substrate concentration, mM. The relation between the sub-maximum substrate concentration and the shifts in the pH optimum observed is considered in the Discussion.

With preparation (B), a pH optimum lower than 3.05 is indicated at a substrate concentration of 0.05 mM, whereas with (A) at this substrate concentration the pH optimum is near 3.6. Possibly the purer preparation (B) is more stable than (A) at lower pH values.

The variation of pK_m with pH is shown in Fig. 6; the results of two separate experiments are in agreement. The regression of pK_m on pH is a line of slope -2.4 . However, the charge change on desubstration (Dixon, 1953) cannot be derived from this variation of pK_m since the pH optimum is so markedly altered with substrate concentration.

DISCUSSION

The preparation of a crude β -glucuronidase from a marine mollusc *Cellana tramoserica* yields products similar to those obtained from other molluscs such as *Patella vulgata* (Dodgson & Spencer, 1953).

Fig. 6. Plot of pK_m against pH. ●, Values of pK_m determined from the data of Fig. 4; ○, determined from the data of Fig. 5A.

The effect of pH, substrate concentration, enzyme concentration, duration and temperature of reaction on the hydrolysis of phenolphthalein glucuronide by this β -glucuronidase follow the pattern generally found with enzymes. From the results with usual procedures, interaction of factors cannot be evident.

The investigation of interactions of these factors showed many highly significant first-, second- and third-order interactions. First-order interactions between pH and temperature, duration of hydrolysis or substrate concentration illustrate the dependence of the 'pH optimum' on the levels of the latter factors. A careful investigation of the action of β -glucuronidase and, in particular, the application of the enzyme to such problems as the hydrolysis of steroid glucuronides should include a survey of interactions between factors; for any particular problem the interactions should be investigated factorially in the working region. Since the β -glucuronidase used showed no abnormal effects in the normal kinetic studies and yet showed significant factor interactions, it is possible that the latter may be of importance for other enzyme reactions.

Factorial experimentation is not necessary for the determination of kinetic constants such as K_m and k_3 , which by definition must be investigated under specified conditions. On the other hand, it is noteworthy that factorial experiments may be arranged to give the data obtainable from experiments where one factor at a time is varied, in addition to the information on factor interactions. The interaction between factors can be determined only by factorial experimentation. In studies such as the investigation of optimum conditions for a reaction, consideration of interactions may be essential to the determination of the optimum.

The shift of the pH optimum with substrate concentration change (Figs. 4, 5) is consistent with a requirement for the participation of un-ionized substrate in the enzymic reaction; at the lower substrate concentrations, the concentration of un-ionized substrate may be the limiting factor and decrease of the pH (above the substrate carboxyl group pK of about 3) would increase the un-ionized substrate concentration. In the pH region investigated only the ionization of the carboxyl group of the glucuronyl radical is concerned. If the shift of the pH optimum is due to the dissociation of the substrate, such shifts should be observed with other β -glucuronides suitable as substrates for β -glucuronidase. Where a marked change in pH optimum with change of substrate concentration occurs, it may be possible to hydrolyse preferentially a selected substrate in a mixture of substrates present in different concentrations.

SUMMARY

1. Kinetics of the hydrolysis of phenolphthalein glucuronide by a molluscan β -glucuronidase preparation were investigated, and in particular the interaction of factors affecting the hydrolysis were studied.

2. Highly significant first-, second- and higher-order factor interactions were found, indicating the importance of factorial experimentation in studies on enzyme reactions.

3. Further examination of one interaction, that between pH and substrate concentration, showed a variation of the pH optimum from 4.8 to 3.6 according to the substrate concentration used.

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