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The Influence of Structure on the Hydrolysis of Substituted Phenyl α-D-Glucosides by α-Glucosidase

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Nath & Rydon (1954) investigated the influence of structure on the hydrolysis of some substituted phenyl β-D-glucosides by β-glucosidase, correlating measured values of the kinetic constants for the enzymic hydrolysis, based on the classical theory of Michaelis & Menten (1913), with the substituent constants (Hammett, 1940), σ, for the substituents in the phenyl group. The present paper describes a similar study of the hydrolysis of some substituted phenyl α-D-glucosides by the α-glucosidase of brewer's yeast. A kinetic investigation of α-glucosidase was carried out by Jsaiev (1926), who studied the hydrolysis of maltose by this enzyme and concluded that Michaelis–Menten kinetics were not strictly obeyed. However, Halvorson & Elias (1958) found that such kinetics were followed in the hydrolysis of *p*-nitrophenyl α-D-glucoside by the α-glucosidase of *Saccharomyces italicus* (K_s = 0·28 mM). The justification for our use of the Michaelis–Menten theory in interpreting our experimental results is discussed below. Substituted phenyl α-D-glucosides are much more difficult to prepare in a state of purity than their β-anomers, and for this reason the present work has involved only 10 substrates, as compared with the 21 studied by Nath & Rydon (1954); the preparation of the substrates is described by Hall, Hollingshead & Rydon (1961).

EXPERIMENTAL

Preparation of α-glucosidase

α-Glucosidase was extracted from brewer's yeast by a modification of the method of Willstätter & Bamann (1926). After autolysis of the yeast with ethyl acetate, α-glucosidase and β-fructofuranosidase were absorbed on alumina gel B and eluted successively with phosphate buffers. The α-glucosidase was eluted with 0·15M-sodium dihydrogen phosphate–disodium hydrogen phosphate buffer, pH 6·9, rather than with the corresponding ammonium system used by the original workers, to avoid interference with the analytical procedure by precipitation of ammonium phosphomolybdate and ammonium phosphotungstate. The stock α-glucosidase solutions so prepared were stored at −25°.

These stock solutions were diluted before use with buffer solution to give enzyme solutions capable of bringing about 30–40% hydrolysis of 0·2 mM-*p*-nitrophenyl α-D-glucoside in 15 min. under standard conditions (see below). The manner in which the dilution was carried out was important. If carried out in one step, much activity was lost in an unpredictable fashion; this was avoided by stepwise dilution, the stock solution being first added to a small volume of buffer and further dilution carried out by adding further amounts of buffer, the solution being gently shaken after each addition, until the required dilution (1:40 to 1:2400, according to the preparation and its age) was achieved. The activity of the diluted solutions increased slightly over a few hours and then remained constant, on storage at 0°, for 2 days; accordingly, dilutions were carried out on the day before a kinetic experiment and the diluted solutions kept overnight at 0°.

Analytical methods

The progress of the hydrolyses was followed by determination of the phenol liberated. The nitrophenols were determined by measurement of the yellow colour developed in dilute NaOH solution (cf. Nath & Rydon, 1954) and the others by the colorimetric method of Folin & Ciocalteu (1927); full details are given by Hollingshead (1958). Control experiments showed that glucose, the phosphate buffer and the diluted enzyme solutions were without effect on the colour development.

Enzymic hydrolyses

The hydrolyses were carried out in test tubes (1 in. × 6 in.) suspended in a bath thermostatically controlled at 35±0·01°. The enzyme and substrate solutions (both in 0·05M-sodium phosphate buffer, pH 6·9) were preheated in the bath for exactly 5 min. before the enzyme was added to the substrate. At suitable times samples were withdrawn, with a preheated pipette, for analysis. With the nitrophenyl glucosides the reaction was stopped by adding 10% (w/v) trichloroacetic acid; in the other cases, the Folin–Ciocalteu reagent itself stopped the reaction.

The optimum pH for the hydrolysis of phenyl α-D-glucoside (0·01M; reaction time 5 min.) was shown to lie between pH 6·85 and 6·95 for two enzyme preparations (see Fig. 1), pH 6·90 being adopted as standard for further work; Jsaiev (1926) gives pH 6·1–6·7 as optimum, Willstätter & Bamann (1926) pH 6·75–7·25, and Halvorson & Elias (1958) 6·6–6·8.

To minimize any possible inhibition by products, the experimental conditions were so adjusted that the amount of hydrolysis in experiments designed for the determination of K_a did not exceed 30%. Initial substrate concentrations were kept as low as possible to avoid zero-order kinetics. The enzyme solutions were very dilute and the reaction velocity was shown to be directly proportional to the

enzyme concentration at the dilutions used. Each single determination of K_a involved following the progress of four separate reactions at four substrate concentrations. Details of one such determination are given in Table 1; details of the others are given by Hollingshead (1958).

The values of k'_{+2} were determined in a separate series of experiments in which the maximum velocity, $V_{max.}$, was determined directly. The same enzyme solution was used for the whole series of substrates at a concentration of 2 mM, which was shown directly in three cases (phenyl, *p*-nitrophenyl and *p*-tolyl) to suffice to give the maximum attainable velocity of hydrolysis for the enzyme concentration used. The reaction time was 7 min. Complete results for all substrates are given by Hollingshead (1958).

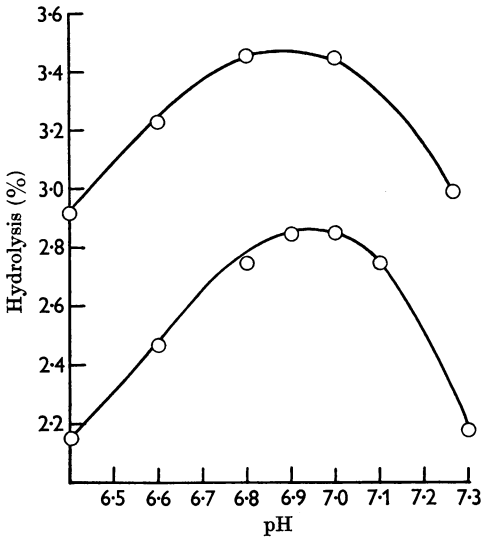

Fig. 1. Optimum pH for hydrolysis of phenyl α-D-glucoside by α-glucosidase.

Calculation of kinetic constants

K_a was evaluated by the procedure of Lineweaver & Burk (1934), as modified by Dixon (1953), in which the reciprocals of the initial velocities, v_0, determined for a series of initial substrate concentrations, a, are plotted against the reciprocals of these concentrations, the negative intercept of the resulting straight line on the horizontal axis being equal to K_a.

Direct determination of v_0 is, of course, not possible, since, however short a time lapse is chosen, the observed velocity will always be less than the true initial velocity. Hence we determined v_0 by the method of Booman & Niemann (1956), in which it is derived from a polynomial fitted to the experimental results. In every case four experimental observations were made at equal time-intervals (see Table 1), giving, with the initial point, a five-point experiment, for which the appropriate polynomials are the following [notation of Booman & Niemann (1956)]:

$$P_{0,4}(s) = 1$$
$$P_{1,4}(s) = 2 - s$$
$$P_{2,4}(s) = 2 - 3s + s(s-1)$$
$$P_{3,4}(s) = 1 - 3s + 2{\cdot}5s(s-1) - 0{\cdot}8333s(s-1)(s-2)$$
$$P_{4,4}(s) = 1 - 5s + 7{\cdot}5s(s-1) - 5{\cdot}8333s(s-1)(s-2) + 2{\cdot}9166s(s-1)(s-2)(s-3)$$

Table 1. *Determination of affinity constant for the hydrolysis of phenyl α-D-glucoside by α-glucosidase*

a, Initial concentration of substrate (moles/l.); t, time (min.); x, concentration of phenol produced (moles/l.); v_0, initial velocity (moles/l./min.); $\bar{v}_0$, mean value of initial velocity (moles/l./min.); K_a, equilibrium constant for formation of enzyme–substrate complex (l./mole). Two other experiments gave K_a values of 1180 and 1386 respectively, leading to a mean value of 1270±60.

Expt. no.	10^5a	t	10^5x (i)	(ii)	(iii)	(iv)	$10^5\bar{v}_0$	K_a
3·1	62·06	5	4·52	4·37	4·52	4·62		
		10	9·70	9·28	9·83	9·90		
		15	14·08	13·87	14·40	14·77		
		20	17·77	17·06	17·77	17·87		
			(10^5v_0 = 1·0426	1·0255	1·0921	1·1149)	1·0688	
3·2	77·58	5	5·15	4·90	5·23	5·15		
		10	10·93	10·86	11·00	11·00		
		15	15·75	15·75	16·00	16·00		
		20	20·88	20·06	20·47	19·35		
			(10^5v_0 = 1·1043	1·1479	1·1651	1·2453)	1·1657	
3·3	93·10	5	5·56	5·08	5·75	5·56		1244
		10	12·43	11·33	11·95	11·95		
		15	18·10	16·35	18·03	16·91		
		20	21·23	20·38	20·88	21·50		
			(10^5v_0 = 1·4463	1·2309	1·4191	1·2796)	1·3440	
3·4	108·61	5	6·83	6·30	6·25	6·12		
		10	12·56	13·02	12·95	12·87		
		15	18·50	18·15	18·90	18·23		
		20	23·54	22·83	23·54	23·85		
			(10^5v_0 = 1·3676	1·4262	1·4215	1·3328)	1·3870	

Table 2. *Kinetic constants for hydrolysis of substituted phenyl α-D-glucosides by α-glucosidase at* pH 6·9 *and* 35°

K_a, Equilibrium constant for the formation of the enzyme–substrate complex (l./mole); k'_{+2} first-order velocity constant for the breakdown of the enzyme–substrate complex (multiplied by an arbitrary constant) (min.$^{-1}$); σ, substituent constant (Hammett, 1940; Mamalis & Rydon, 1955). Kinetic constants are mean values ± S.E., with the numbers of determinations in parentheses.

Substituent	K_a	k'_{+2}	σ
H	1270± 60 (3)	7·55±0·24 (4)	0·000
o-Me	3968±126 (2)	9·51±0·05 (3)	−0·054
m-Me	1798±366 (3)	10·27±0·04 (4)	−0·069
p-Me	1496± 29 (2)	6·21±0·02 (4)	−0·170
m-MeO	2095± 75 (3)	10·40±0·12 (4)	+0·115
p-MeO	1820± 21 (2)	5·02±0·01 (3)	−0·268
m-Cl	3885±249 (2)	13·12±0·13 (4)	+0·373
p-Cl	2229±162 (3)	8·40±0·07 (4)	+0·227
m-NO_2	3097±217 (2)	11·89±0·17 (3)	+0·210
p-NO_2	6272±265 (4)	7·48±0·14 (3)	+1·270

Further mathematical details of the calculations are given by Hollingshead (1958). In general, the experimental results were satisfactorily fitted by an equation of the second degree. In one case (*o*-tolyl α-D-glucoside) a direct comparison was made between a five-point and an eight-point experiment; the agreement between the individual values of v_0 was no better for the eight-point experiment.

Unless a pure enzyme of known molecular weight is available, it is not possible to determine the absolute value of the first-order velocity constant, k_{+2}, for the breakdown of the enzyme–substrate complex, since the expression, $k_{+2} = V_{max.}/e$, used for the evaluation of k_{+2} involves (as do all other expressions for evaluating k_{+2}) the molar enzyme concentration, e. For comparative purposes (cf. Nath & Rydon, 1954; Hall, Leeson, Rydon & Tweddle, 1960), k'_{+2}, the velocity constant multiplied by a constant but unknown factor, suffices; in the present work e was constant in all the experiments used for evaluating v_0 and was assigned the arbitrary value of unity.

RESULTS

The results (Table 2) are expressed in terms of the affinity constant, K_a (i.e. k_{+1}/k_{-1}), for the formation of the enzyme–substrate complex, and of the first-order velocity constant, k_{+2}, for the decomposition of this complex, according to the Michaelis–Menten scheme:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} E + P$$

As in previous studies (Nath & Rydon, 1954; Hall *et al.* 1960; cf. Laidler, 1958*a*) we prefer K_a, as being a more direct measure of enzyme–substrate affinity, to its more commonly used reciprocal, K_s, the 'substrate constant'.

DISCUSSION

Before attempting to correlate the kinetic constants with the structures of the substrates we must inquire more deeply into their significance, since, although there is no doubt about the meaning of k_{+2}, this is not true of K_a. According to the strict, equilibrium, treatment of Briggs & Haldane (1925), K_a, as evaluated from the experimental results, is equal to $k_{+1}/(k_{-1}+k_{+2})$ and only becomes equal to the required equilibrium constant, k_{+1}/k_{-1}, if $k_{+2} \ll k_{-1}$; at the other extreme, when $k_{+2} \gg k_{-1}$, the formulation of Van Slyke & Cullen (1914) applies and $K_a = k_{+1}/k_{+2}$. Various tests (Laidler, 1958*b*) have been suggested to decide between these possibilities, but none are readily applicable to our work.

However, in studies such as ours, in which the kinetic constants are evaluated for a range of substituted substrates to which the Hammett (1940) equation is applicable, the point can be settled in another way. If $K_a = k_{+1}/k_{-1}$, then it is to be expected that a plot of log K_a against the Hammett substituent constant, σ, will be linear; on the other hand, if $K_a = k_{+1}/k_{+2}$, then $K_a k_{+2} = k_{+1}$, and it is to be expected that a plot of log $(K_a k_{+2})$ against σ will be linear. In the present instance the former plot (Fig. 2) is satisfactorily linear, the correlation coefficient, r, being 0·80, and the S.D. 0·29, both values falling within the limits proposed by Jaffé (1953) for satisfactory correlations of this kind; on the other hand the similar plot of log $(K_a k_{+2})$ against σ is not satisfactorily linear (r, 0·61; S.D., 1·20). Clearly our measured values for K_a approach k_{+1}/k_{-1} more nearly than they do k_{+1}/k_{+2}. Further, if the experimentally determined K_a has the composite value $k_{+1}/(k_{-1}+k_{+2})$, it would be expected that the points for substrates showing high values of k_{+2} would deviate below the regression line in the plot of log K_a against σ, since a high k_{+2} will lower the value of a composite K_a, and those for substrates with low values of k_{+2}, above the line; inspection of Fig. 2 shows that this is not the case with our results, and we therefore conclude that our measured K_a values approximate to the true equilibrium constants, k_{+1}/k_{-1}.

Fig. 2 shows that, as in the β-series (Nath & Rydon, 1954), the affinity of the substrate for the enzyme is greater when electron-attracting substituents are present in the phenyl group, although the effect is less marked, the reaction constant, ρ (Hammett, 1940), having the value +0·41, as against +0·95 for the β-series. As in the β-series, too, the stereochemical specificity of the enzyme requires at least three centres to be involved in the attachment of the substrate to the enzyme (Rydon, 1948). The very low value of the reaction constant for the α-glucosides is difficult to reconcile with simple facilitation of attachment to the enzyme by electron-attracting substituents in the phenyl group; it suggests the operation of two opposing electronic influences, one resulting in facilitation and the other hindrance of attachment by electron-attracting substituents. A possible modification of the mode of attachment for the β-glucosides, suggested by Nath & Rydon (1954) to accommodate this, is shown in Fig. 3; it involves (*a*) hydrogen bonding through hydroxyl groups of the glucose moiety, possibly to an imidazole group in the enzyme (Larner & Gillespie, 1956; Larner, 1960), (*b*) hydrogen bonding through the glucosidic oxygen, and (*c*) van der Waals interaction with the phenyl group. Attachment to the enzyme side chains —X through the hydrogen atoms of the 2- and 3- (and perhaps other) hydroxyl groups will be facilitated by electron-attracting substituents, whereas attachment of the side chain —YH through the glucosidic oxygen atom will be hindered by such substituents. The axial position of the glucosidic oxygen (*cis-* to the 2-hydroxyl group) in the α-glucosides makes its participation in such a hydrogen bond easier than for the β-glucosides, in which this oxygen (*trans-* to the 2-hydroxyl group) is equatorial; this difference between the two series is best appreciated by inspection of models.

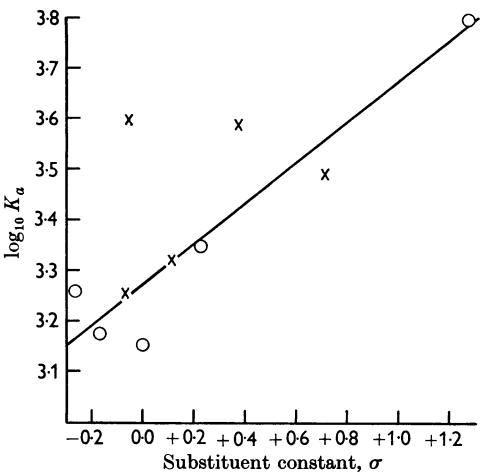

Fig. 2. Correlation of K_a with structure. ○, Substrates for which $k'_{+2} < 9{\cdot}0$; ×, substrates for which $k'_{+2} > 9{\cdot}0$. The line was drawn by the method of least squares, neglecting the point for the *o*-tolyl compound.

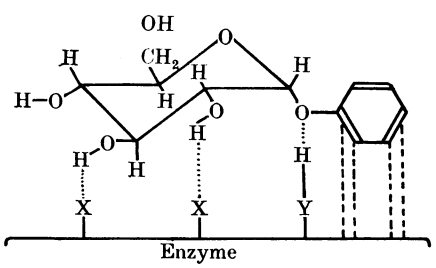

Fig. 3. Possible mode of attachment of phenyl α-D-glucoside to α-glucosidase. ···, Hydrogen bond; - - -, van der Waals interaction.

The considerable facilitation of attachment to the enzyme brought about by the introduction of an *o*-methyl substituent is noteworthy, but cannot usefully be commented on in the absence of information on other *ortho*-substituted compounds.

When log k'_{+2} is plotted against σ the points are very scattered and, although there is some indication of facilitation of breakdown of the enzyme–substrate complex by the introduction of electron-attracting substituents, as in the β-series, no satisfactory correlation can be achieved, however the points are grouped; for this reason no useful comment is possible.

SUMMARY

1. The hydrolysis by α-glucosidase of phenyl α-D-glucoside and nine of its substitution products has been studied; the equilibrium constants for the formation of the enzyme–substrate complexes and the first-order velocity constants for their decomposition have been evaluated.

2. A new method for assessing the significance of experimentally determined enzyme–substrate equilibrium constants is proposed.

3. The equilibrium constants have been correlated with the electronic properties of the substituents, as measured by their substituent constants, and a mechanism for the combination of α-glucosidase with aryl α-D-glucosides is suggested.

4. No similar correlation of the velocity constants with structure was possible.

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Effects of Ouabain on Cerebral Metabolism and Transport Mechanisms *in vitro*

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This work is part of a study of the effects of metabolic inhibitors and neurotropic agents on the formation of amino acids from glucose in the isolated rat-brain cortex, some results of which have been reported (Gonda & Quastel, 1961). We have examined the action of ouabain, a drug well known for its inhibitory effects on electrolyte transport at the cell membrane (Schatzmann, 1953; Kahn & Acheson, 1955; Glynn, 1957; Caldwell & Keynes, 1959). This substance has remarkable effects on the transformation of glucose into amino acids in isolated rat-brain slices and on amino acid and creatine transport into brain *in vitro*. This paper describes results that have been obtained and their possible interpretation.

MATERIALS AND METHODS

The method for the study of the conversion of radioactive glucose into radioactive amino acids was based on that described by Kini & Quastel (1959). The Warburg manometric apparatus was used for incubation of brain-cortex slices in oxygen at 37°. Brain-cortex slices, from adult hooded rats of about 150 g., were placed in ice-cold Krebs–Ringer phosphate medium of the following composition: NaCl, 128 mM; KCl, 5 mM; $CaCl_2$, 3·6 mM; $MgSO_4$, 1·3 mM; Na_2HPO_4, 10 mM (brought to pH 7·4 with N-HCl). For study of the effects of high K^+ ion concentrations, the KCl concentration in the medium was increased to 105 mM, the concentrations of the other ions remaining the same. The slices were prepared with a Stadie–Riggs microtome, care being taken that the slices were not more than 0·3 mm. thick, but not so thin that they tended to disintegrate when shaken in a Warburg manometric apparatus. The slices (average wet wt. 80–90 mg.) were weighed at once on a torsion balance and suspended in the incubation medium in chilled manometric vessels. The dry wt. of the slices prepared under these conditions was approximately 16% of the wet wt. The final volume of medium in each Warburg manometric flask was 1 ml. and each flask contained 5 mM-glucose uniformly labelled with ^{14}C. Corrections were not made for swelling of the tissue. This was variable and did not exceed 10%.

After aerobic incubation for 1 hr. at 37°, the flasks were chilled in crushed ice, and the slices were removed, rinsed with a small quantity of Krebs–Ringer phosphate medium, suspended in 5 ml. of 80% (v/v) ethanol and homogenized in a Potter–Elvehjem homogenizer. The homogenizer was washed with small portions of 80% ethanol and the washings were added to the original homogenate. The homogenate was kept at 5° for 2 hr., then centrifuged; the ethanolic extract was collected and the insoluble residue washed twice with 1·5 ml. portions of 80% ethanol and finally with