The Influence of Structure on the Hydrolysis of Substituted Phenyl β -D-glucosides by Emulsin

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Detailed information on the mode of interaction of an enzyme with its substrate can probably best be obtained by correlating enzyme activity with structural changes in the substrate. Such information is of interest both in itself and as a basis for chemotherapeutic studies, and the present paper is the first of a series in which it is proposed to study a number of enzyme reactions from this point of view.

The enzyme emulsin was chosen for this initial study since it is comparatively unspecific, catalysing the hydrolysis of a wide range of glycosides. The group of substrates chosen for the work was that of the phenyl β -D-glucosides, in which structural changes can readily be brought about by the introduction of substituents into the benzene ring; the electronic effects of such substituents can be measured quantitatively by means of the substitution constants (σ values) of Hammett (1938, 1940). Following the work of Josephson (1925), the action of emulsin on various glucosides has been extensively studied by Helferich & Veibel and their colleagues (for refs. see Pigman, 1944, and Pigman & Goepf, 1948); this large volume of work has not, however, led to any very clear conclusions, partly because the structural variation in the aglucones has not been sufficiently systematic and partly because of variations in the experimental conditions, but mainly because, in most cases, the results have been expressed only in terms of the overall velocity constant of the reaction. According to the widely accepted views of Michaelis & Menten (1913), the action of an enzyme on a substrate falls into two distinct stages, namely, reversible combination to form an enzyme-substrate complex and irreversible breakdown of this complex into regenerated enzyme and products; structural changes in the substrate will not necessarily have similar effects on these two stages of the overall reaction and it is therefore desirable to obtain a quantitative measure of both, the first in terms of its equilibrium constant (the reciprocal of the usual Michaelis constant) and the second in terms of its velocity constant.

We describe below the determination of both of these constants for the emulsin-catalysed hydrolysis of phenyl β -D-glucoside and twenty of its sub-

stitution products, together with the determination of the velocity constants for the acid- and alkali-catalysed hydrolyses of the same series of glucosides.

EXPERIMENTAL

Preparation of the glucosides

(Light petroleum denotes the fraction of b.p. 60–80°.)

The glucosides were all prepared by way of their tetra-acetyl derivatives, most of which were prepared by the following procedure (cf. Helferich & Schmitz-Hillebrecht, 1933; Sisido, 1936). The phenol (0.3–0.4 mole) was heated with penta-O-acetyl- β -D-glucose (0.1 mole; Fischer, 1916) and *p*-toluenesulphonic acid (0.7–2.0 g.) under reduced pressure at 100–120° for 1 hr. 30 min. to 2 hr. The cooled product was dissolved in benzene (200 ml.) and the solution washed with water and then repeatedly with 10% (w/v) NaOH soln. until the washings were colourless. The solution was again washed with water, dried (CaCl₂) and evaporated under reduced pressure. The residue was crystallized from methanol using charcoal; this crude product was then crystallized to constant m.p. from a suitable solvent. In certain cases (*o*-tert.-butyl, 2,6-dimethyl and *p*-methoxy) it was found advantageous to run the washed benzene extract through a column of alumina, eluting with more benzene, in order to remove dark, alkali-insoluble impurities.

In four cases (the *o*-nitro- and *o*-, *m*- and *p*-cyano compounds) the method of Robertson & Waters (1930) was used, thus: acetobromoglucose (0.05 mole; Fischer, 1916), the requisite phenol (0.025 mole) and dry, redistilled quinoline (about 50 ml.) were made into a paste with which dry, freshly prepared 'active' silver oxide (0.05 mole; Helferich & Klein, 1926) was thoroughly triturated. The mixture was kept in a desiccator for 1 hr. 30 min., stirring every 15 min., and then extracted with glacial acetic acid (450 ml.) at room temp. and filtered. The filtrate was poured slowly, with mechanical stirring, into water (4 l.) at 0° and the precipitate either collected and directly crystallized or (for the *o*- and *m*-cyano compounds) chromatographed on alumina in benzene/light petroleum, eluted with benzene and crystallized.

The tetra-acetylglucosides were deacetylated (Zemplen & Pacsu, 1929) by dissolving in anhydrous methanol (200–800 ml. for 25 g.) and adding a freshly prepared solution of sodium methoxide (from sodium, 10–20 mg., and methanol, 10 ml.). The mixture was heated on the steam bath for 6–8 min. and then kept at room temp. overnight. The methanol was then removed under reduced pressure and the residual glucoside crystallized to constant m.p. using, if possible, more than one solvent.

The properties of the glucosides used were as follows:

Phenyl β -D-glucoside, crystallized from water, m.p. 171–172° after drying at 130–140° *in vacuo*. (Found: C, 56.5; H, 6.4. Calc. for $C_{12}H_{16}O_6$: C, 56.3; H, 6.25%.) Fischer & Armstrong (1901) give m.p. 174–175° for the anhydrous compound, Robertson & Waters (1930), m.p. 176° for a monohydrate, and Helferich & Schmitz-Hillebrecht (1933), m.p. 144–145° for a dihydrate.

o-Cresyl β -D-glucoside, crystallized from water and ethanol/benzene, m.p. 162–163°; Helferich & Scheiber (1934) give m.p. 163–164°.

m-Cresyl β -D-glucoside, crystallized from water, m.p. 180°; Helferich & Philipp (1934) give m.p. 183–184°.

p-Cresyl β -D-glucoside, crystallized from water and ethanol/benzene, m.p. 179–180°; Helferich & Scheiber (1934) give m.p. 178–179°.

o-isoPropylphenyl β -D-glucoside, was very hygroscopic and resisted all attempts at recrystallization; the product, dried at 100° *in vacuo* over P_2O_5 , had m.p. 60–70°, $[\alpha]_D^{25}$ –63.8° in water (c, 0.6). (Found: C, 60.2; H, 7.5. $C_{15}H_{22}O_6$ requires C, 60.4; H, 7.4%.) The *tetra-acetyl derivative* crystallized from benzene in prismatic needles, m.p. 195–196°, $[\alpha]_D^{22}$ –19.0° in $CHCl_3$ (c, 2.05). (Found: C, 59.5; H, 6.8. $C_{23}H_{30}O_{10}$ requires C, 59.2; H, 6.4%.)

p-isoPropylphenyl β -D-glucoside crystallized from water and ethanol/light petroleum in plates, m.p. 160–161°, $[\alpha]_D^{21}$ –50.2° in ethanol (c, 1.28). (Found: C, 59.8; H, 7.6. $C_{15}H_{22}O_6$ requires C, 60.4; H, 7.4%.) The *tetra-acetyl derivative* crystallized from methanol in plates, m.p. 177–179°, $[\alpha]_D^{22}$ –18.2° in $CHCl_3$ (c, 1.15). (Found: C, 59.4; H, 6.7. $C_{23}H_{30}O_{10}$ requires C, 59.2; H, 6.4%.)

o-tert-Butylphenyl β -D-glucoside was very hygroscopic and resisted all attempts at recrystallization; the product, dried at 100° *in vacuo* over P_2O_5 , had m.p. 70–90°, $[\alpha]_D^{23}$ –64.1° in water (c, 0.62). (Found: C, 60.5; H, 7.9. $C_{16}H_{24}O_6$ requires C, 61.5; H, 7.7%.) The *tetra-acetyl derivative* crystallized from ethanol in plates, m.p. 191°, $[\alpha]_D^{22}$ –34.8° in $CHCl_3$ (c, 1.32). (Found: C, 60.1; H, 6.8. $C_{24}H_{32}O_{10}$ requires C, 60.0; H, 6.7%.)

p-tert-Butylphenyl β -D-glucoside, crystallized from ether/light petroleum, m.p. 145–146°; Helferich, Scheiber & Hiltmann (1940) give m.p. 148–149.5°.

2,4-Dimethylphenyl β -D-glucoside, crystallized from ethyl acetate/light petroleum and water, m.p. 175–176°, $[\alpha]_D^{21}$ –72.4° in water (c, 0.58). (Found: C, 59.0; H, 7.6. Calc. for $C_{14}H_{20}O_6$: C, 59.2; H, 7.05%.) Helferich, Scheiber, Streeck & Vorsatz (1935) give m.p. 181–183.5°, $[\alpha]_D^{20}$ –65°. The *tetra-acetyl derivative*, crystallized from ethanol/light petroleum, had m.p. 122–123°, $[\alpha]_D^{22}$ –18.8° in $CHCl_3$ (c, 1.6). (Found: C, 58.6; H, 6.3. Calc. for $C_{22}H_{28}O_{10}$: C, 58.4; H, 6.2%.) Helferich *et al.* (1935) give m.p. 171–173°, which appears to be in error, and $[\alpha]_D$ –20°.

2,6-Dimethylphenyl β -D-glucoside, crystallized from water, m.p. 209–211°; Helferich & Scheiber (1934) give m.p. 209–211°.

o-Methoxyphenyl β -D-glucoside, crystallized from water as the monohydrate, m.p. 155–156° (Helferich & Burt, 1935, give m.p. 155.5–157.5°), which became anhydrous after 2 hr. *in vacuo* at 110–120°. (Found: C, 54.1; H, 6.5. Calc. for $C_{13}H_{18}O_7$: C, 54.5; H, 6.3%.)

m-Methoxyphenyl β -D-glucoside, crystallized from water and ethanol/light petroleum in needles, m.p. 159–161°, $[\alpha]_D^{18}$ –69.2° in water (c, 1.04). (Found: C, 54.9; H, 6.45.

$C_{13}H_{18}O_7$, requires C, 54.5; H, 6.3%.) The *tetra-acetyl derivative* crystallized from ethanol in plates, m.p. 130–131°, $[\alpha]_D^{15}$ –20.6° in $CHCl_3$ (c, 3.2). (Found: C, 55.8; H, 5.95. $C_{21}H_{26}O_{11}$ requires C, 55.5; H, 5.7%.)

p-Methoxyphenyl β -D-glucoside, crystallized from water, m.p. 175–177°; Helferich & Schmitz-Hillebrecht (1933) give m.p. 175–177°.

o-Chlorophenyl β -D-glucoside, crystallized from water and ethanol in needles, m.p. 170–171°, $[\alpha]_D^{18.5}$ –90.6° in water (c, 0.73). (Found: C, 49.7; H, 5.4. $C_{13}H_{15}O_6Cl$ requires C, 49.6; H, 5.2%.) The *tetra-acetyl derivative* crystallized from benzene/light petroleum in prisms, m.p. 149–150°, $[\alpha]_D^{16}$ –51.6° in $CHCl_3$ (c, 1.45). (Found: C, 51.65; H, 5.2. $C_{20}H_{23}O_{10}Cl$ requires C, 52.4; H, 5.0%.)

m-Chlorophenyl β -D-glucoside, crystallized from ethanol, ethyl acetate and water in prismatic needles, m.p. 179–180°, $[\alpha]_D^{16}$ –36.3° in water (c, 0.59). (Found: C, 49.8; H, 5.4. $C_{13}H_{15}O_6Cl$ requires C, 49.6; H, 5.2%.) The *tetra-acetyl derivative* crystallized from methanol in needles, m.p. 131.5–133.5°, $[\alpha]_D^{16}$ –22.8° in $CHCl_3$ (c, 1.79). (Found: C, 52.4; H, 5.1. $C_{22}H_{25}O_{10}Cl$ requires C, 52.4; H, 5.0%.)

p-Chlorophenyl β -D-glucoside, crystallized from water, m.p. 173–175°, $[\alpha]_D^{19}$ –82.0° in water (c, 1.13). (Found: C, 49.6; H, 5.4. Calc. for $C_{13}H_{15}O_6Cl$: C, 49.6; H, 5.2%.) Dyfverman & Lindberg (1950) give m.p. 173–174°, $[\alpha]_D^{20}$ –69.5°.

o-Cyanophenyl β -D-glucoside crystallized from ethyl acetate/light petroleum in prisms, m.p. 176–177°, $[\alpha]_D^{18.5}$ –70.9° in water (c, 1.55). (Found: C, 55.6; H, 5.6. $C_{13}H_{15}O_6N$ requires C, 55.5; H, 5.35%.) The *tetra-acetyl derivative* crystallized from ethanol, m.p. 163–164°, $[\alpha]_D^{18}$ –14.2° in $CHCl_3$ (c, 2.28). (Found: C, 56.2; H, 5.25; N, 3.1. $C_{21}H_{23}O_{10}N$ requires C, 56.1; H, 5.1; N, 3.1%.)

m-Cyanophenyl β -D-glucoside, crystallized from water, ethyl acetate/light petroleum, ethanol/light petroleum and ethanol in fine prisms, m.p. 167–168°, $[\alpha]_D^{18.5}$ –137.7° in water (c, 1.59). (Found: C, 56.2; H, 5.6. $C_{13}H_{15}O_6N$ requires C, 55.5; H, 5.35%.) The *tetra-acetyl derivative* crystallized from ethanol in prismatic needles, m.p. 126–127°, $[\alpha]_D^{18}$ –31.5° in $CHCl_3$ (c, 2.34). (Found: C, 56.45; H, 5.3. $C_{21}H_{23}O_{10}N$ requires C, 56.1; H, 5.1%.)

p-Cyanophenyl β -D-glucoside, crystallized from water in long prisms, m.p. 193–194°, $[\alpha]_D^{18.5}$ –91.9° in water (c, 1.51). (Found: C, 55.6; H, 5.6. $C_{13}H_{15}O_6N$ requires C, 55.5; H, 5.35%.) The *tetra-acetyl derivative* crystallized from methanol in long prisms, m.p. 151–153°, $[\alpha]_D^{18.5}$ –29.4° in $CHCl_3$ (c, 2.90). (Found: C, 56.1; H, 5.2; N, 3.35. $C_{21}H_{23}O_{10}N$ requires C, 56.1; H, 5.1; N, 3.1%.)

o-Nitrophenyl β -D-glucoside crystallized from water as a hydrate, which lost its water at 110° over P_2O_5 *in vacuo* and then had m.p. 168–170°, $[\alpha]_D^{18.5}$ –140.4° in water (c, 2.14). (Found: C, 48.2; H, 5.25. Calc. for $C_{12}H_{15}O_6N$: C, 47.8; H, 5.0%.) Montgomery, Richtmyer & Hudson (1942) give m.p. 152°, $[\alpha]_D$ –106°, for the anhydrous compound, while Glaser & Wulwek (1924) give m.p. 130–131°, $[\alpha]_D^{14}$ –82.7°, for the hydrate.

m-Nitrophenyl β -D-glucoside, crystallized from water and dried *in vacuo* at 110°, had m.p. 166–168°, $[\alpha]_D^{18.5}$ –87.3° in water (c, 1.92). (Found: C, 47.4; H, 5.2. Calc. for $C_{12}H_{15}O_6N$: C, 47.8; H, 5.0%.) Glaser & Wulwek (1924) give m.p. 167–168°, $[\alpha]_D^{22}$ –84.9°, for the monohydrate.

p-Nitrophenyl β -D-glucoside, crystallized from water, m.p. 164–165°; Glaser & Wulwek (1924) give m.p. 164–165°.

Analytical methods

In most previous work on the hydrolysis of glucosides by emulsin the reaction was followed polarimetrically, a sample of the reaction mixture being withdrawn at an appropriate time, added to saturated K_2CO_3 solution, allowed to mutarotate and examined in the polarimeter. Although simple, this method suffers, as Veibel & Eriksen (1936) have pointed out, from serious disadvantages; thus, allowance has to be made for the initial rotation of the reaction mixture and this is determined at the reaction pH (4.4–5.0), whereas the final rotation is determined at pH 10.5–10.6, at which the specific rotations of glucose and the glucosides differ significantly from those at the lower pH; moreover, appreciable decomposition of glucose occurs during the period allowed for mutarotation. Although suitable corrections can be made, we have preferred to determine either the glucose or the phenol produced by the hydrolysis by direct chemical methods, thus obviating these uncertainties.

The enzymic hydrolyses of all but the cyano- and nitrophenyl glucosides were followed by estimating the glucose formed using a modification of the method of Jendrassik & Polgar (1940) (cf. Fujita & Iwatake, 1931). The procedure was as follows (the various solutions are those described by Jendrassik & Polgar, 1940): 1 ml. of the glucose solution was mixed with 0.8 ml. of 0.1N-NaOH and 0.5 ml. of cadmium sulphate solution. The mixture was heated in a boiling-water bath for 3 min. and filtered through a plug of washed cotton wool, which was then thoroughly washed with hot water. To the filtrate and washings, 2 ml. of alkaline ferricyanide solution were added and the mixture was heated in a boiling-water bath for 15 min., cooled and titrated with 0.005N- $Na_2S_2O_3$, after adding 0.5 ml. of acid $ZnSO_4$ solution and a few drops of starch-iodide solution. A blank was carried out simultaneously using 1 ml. of 0.005M acetate buffer, pH 5.25, in place of the glucose solution. If a (ml.) is the titre of the glucose solution and b that of the blank, the molar concentration of glucose is $0.000965 f(b-a)$, where f is the factor of the $Na_2S_2O_3$. In twenty control experiments, using standard glucose solutions, the error fell between +1.0% and -3.0%. The same method, except that the cadmium sulphate treatment was omitted and the solution was neutralized with the calculated amount of 0.01N-NaOH before adding the alkaline ferricyanide, was used for following the acid-catalysed hydrolyses of the same glucosides; the error in the control experiments fell within the limits +0% and +3.5%.

This method failed for the cyano compounds, and the enzyme- and acid-catalysed hydrolyses of the *m*- and *p*-cyanophenyl glucosides were followed by estimating the liberated glucose in 1 ml. samples by the method of Folin & Wu (1920) (cf. Hawk, Oser & Summerson, 1947*a*). The final colour was measured in a 1 cm. cell, against a water blank, with a Spekker Photoelectric Absorptiometer using an Ilford Spectrum Red filter, no. 608 (wavelength of peak transmission, 680 m μ); a standard curve covering the range up to 9 mM glucose (Spekker reading, 0.65) was used. The method was unsatisfactory for the *o*-cyanophenyl glucoside.

The alkali-catalysed hydrolyses of all but the nitrophenyl glucosides were followed by estimation of the liberated phenols by the method of Folin & Ciocalteu (1927) (cf. Hawk, Oser & Summerson, 1947*b*); methods based on the estima-

tion of glucose cannot be used, owing to the instability of glucose in alkaline solution, while the polarimetric method employed by Dyfverman & Lindberg (1950) was not applicable to our more dilute solutions. The final colour was measured in a 1 cm. cell with a Spekker Photoelectric Absorptiometer, using an Ilford Spectrum Red filter, no. 608 (680 m μ); reference curves were constructed for each phenol covering the range up to 1.4 mM (Spekker reading, 0.3–0.9).

The nitrophenols were estimated in enzymic, acid and alkaline hydrolysates by means of their yellow colour in alkaline solution. The reaction mixture (1 ml.) was neutralized if necessary and so diluted with water and NaOH solution as to give a final NaOH concentration of 0.01N for the *o*- and *p*-compounds and 0.033N for the *m*-compound. The yellow colour was measured in a 1 cm. cell with a Spekker photoelectric absorptiometer using an Ilford Spectrum Violet filter, no. 601 (wavelength of peak transmission, 430 m μ). Reference curves were made for each phenol, covering the range up to 16×10^{-5} M (Spekker readings, 0.194, 0.210 and 0.655 for *o*-, *m*- and *p*-nitrophenols respectively).

Preparation of emulsin

The enzyme was prepared from sweet almond meal by the tannin precipitation method of Helferich, Winkler, Gootz, Peters & Günther (1932); the alcohol-extraction procedure of Tauber (1932) gave a much less active product. The product (the 'Rohferment' of Helferich *et al.* 1932) was washed free of tannin (no blue colour with $FeCl_3$) with acetone and dried over H_2SO_4 *in vacuo* at 0° for 3 days. After equilibration with atmospheric moisture it was preserved at 0° over silica gel. The yield of material containing 12.1% nitrogen was 1.4%, based on the sweet almond meal used.

β -D-Glucosidase activity is conveniently expressed as the Ph.f. value (cf. the Sal.f. value used by Josephson, 1925, Weidenhagen, 1929, and Helferich, Gootz & Sparmberg, 1932) which we define as the first-order velocity constant (min.⁻¹) for the hydrolysis of phenyl β -D-glucoside in 0.05M concentration at 30° and pH 5.25 calculated for a standard enzyme concentration of 100 mg./l. (the reaction was shown experimentally to be first order with respect to the enzyme). The Ph.f. value of the sample used for all our work changed negligibly during the course of the work, being 4.44 at the beginning (March, 1950) and 4.28 at the end (June, 1951). A Ph.f. value of 4.44 corresponds to an 'enzyme value' ('Wertigkeit') (Helferich, 1938) of 0.30 and may be compared with Helferich's value of 0.33 for his preparation of 'Rohferment'.

A small amount of the standard emulsin was further purified by the silver precipitation method of Helferich & Winkler (1932); the enzyme from the second precipitate

Table 1. Comparison of equilibrium constants using 'Rohferment' and silver-purified enzyme

β -D-Glucoside	Equilibrium constant, K_a	
	'Rohferment'	Silver-purified enzyme
Phenyl	36.75	32.25
<i>o</i> -Cresyl	43.4	50.75
<i>m</i> -Cresyl	52.95	42.4
<i>p</i> -Cresyl	76.9	71.95

(2.3% yield) had Ph.f. = 20.8 and that from the third precipitate (0.16% yield) Ph.f. = 26.0. As shown in Table 1, this further purification had no very great effect on the equilibrium constants and, owing to the very poor yield of the silver-purified enzyme, we therefore used our 'Roh-ferment' preparation for enzymic hydrolysis, the same sample being used throughout.

Optimum conditions for enzymic hydrolysis

Veibel & Lillelund (1940*b*) studied the effect of pH on the hydrolysis of a number of glucosides with emulsin and found that the overall velocity constant was maximal at pH 5.0 for *o*-cresyl β -D-glucoside and at pH 4.4 for *n*-butyl β -D-glucoside; they further found that, for *o*-cresyl β -D-glucoside, the equilibrium constant was greatest at pH 5.0–5.2. We determined the overall velocity constant for the hydrolysis of nine of our glucosides at pH 4.5 and 5.0 (see Table 2)

Table 2. *Velocity constants ($10^4 k_0$; min.⁻¹) for hydrolysis of β -D-glucosides with emulsin (100 mg./l.) at 30°*

Substrate concentration, 0.01M; 0.01M acetate buffer.

β -D-Glucoside	pH 4.5	pH 5.0
Phenyl	4.34*	4.42*
<i>o</i> -Cresyl	321	342
<i>m</i> -Cresyl	20.5	23.4
<i>o</i> -Methoxyphenyl	322	431
<i>m</i> -Methoxyphenyl	37.1	44.5
<i>p</i> -Methoxyphenyl	23.8	29.1
<i>o</i> -Chlorophenyl	487	643
<i>m</i> -Chlorophenyl	144.5	176.5
<i>p</i> -Chlorophenyl	24.3	27.0

* 0.05M substrate.

Table 3. *Hydrolysis of phenyl β -D-glucoside by emulsin at 30° in 0.005M acetate buffer, pH 5.25*

e'_0 = Enzyme concentration, mg./l.

s_0 = Substrate concentration, moles/l.

t = Time, min.

a = $\text{Na}_2\text{S}_2\text{O}_3$ titre of 1 ml. of reaction mixture, ml.

b = $\text{Na}_2\text{S}_2\text{O}_3$ titre of 1 ml. enzyme blank, ml.

f = Factor of 0.005N- $\text{Na}_2\text{S}_2\text{O}_3$.

x = Glucose concentration, moles/l.

k_0 = Overall velocity constant, min.⁻¹.

k'_3 = First-order velocity constant for decomposition of enzyme-substrate complex, min.⁻¹.

K_a = Equilibrium constant for formation of enzyme-substrate complex, l./mole.

Expt. no.	e'_0	s_0	f	b	t	a	$10^4 x$	$10^4 k_0$	$10^4 k'_3$	K_a
46	100	0.008	0.779	2.48	30	2.26	1.65	6.37	24.2	
					60	2.06	3.16			
					90	1.89	4.44			
47	100	0.016	0.779	2.48	30	2.11	2.78	5.41	24.8	33.3
					60	1.78	5.26			
					90	1.47	7.60			
48	100	0.024	0.779	2.48	30	2.03	3.39	4.55	24.6	
					60	1.60	6.62			
					90	1.20	9.63			
49	75	0.006	0.779	2.48	45	2.27	1.58	5.10	21.8	
					90	2.09	2.93			
					135	1.95	3.98			
50	75	0.008	0.779	2.48	45	2.21	2.03	4.81	21.8	
					90	1.99	3.69			
					135	1.81	5.04			
51	75	0.012	0.779	2.48	45	2.14	2.56	4.35	22.0	38.5
					90	1.83	4.89			
					135	1.57	6.84			
52	75	0.016	0.779	2.48	45	2.03	3.39	3.86	21.6	
					90	1.73	5.64			
					135	1.38	8.27			
53	42.5	0.008	0.774	2.48	80	2.25	1.72	2.70	21.6	
					160	2.05	3.21			
					240	1.80	5.08			
54	42.5	0.012	0.774	2.48	80	2.17	2.32	2.40	21.5	38.5
					160	1.88	4.48			
					240	1.58	6.73			
55	42.5	0.016	0.774	2.48	80	2.11	2.76	2.18	21.5	
					160	1.73	5.61			
					240	1.40	8.07			
							Mean values		22.5	36.75
							S.E.		+0.4	+1.7

and found that, in every case, the reaction was faster at pH 5.0; further experiments with five glucosides showed that the velocity constant was almost unaffected by change of pH over the range 5.0–5.5. We therefore adopted pH 5.25 for our work.

Veibel & Lillelund (1940b) also showed that acetate buffer had some inhibiting effect on the hydrolysis of *o*-cresyl β -D-glucoside; we therefore chose a low buffer concentration, namely 0.005 M.

All our enzymic hydrolyses were carried out at $30^\circ \pm 0.05^\circ$ and, in order to avoid the necessity for correcting for interference by the reaction products (cf. Veibel & Eriksen, 1934–6), the enzyme and substrate concentrations were so chosen that the degree of hydrolysis was never more than 20% (in 90% of the experiments it was less than 10%).

Enzymic hydrolyses

The hydrolyses were carried out in 25 ml. stoppered flasks into which were introduced suitable volumes of acetate buffer (prepared according to Green, 1933, and checked by means of a Cambridge pH meter), glucoside solution, water and enzyme solution to give a total volume of 10 ml., with a buffer concentration of 0.005 M and the desired final concentrations of glucoside and enzyme; the enzyme solution, prepared by dissolving a weighed amount in a little water, filtering from a small amount of insoluble material and making up to volume with water, was added last, after preheating the other components in the thermostat for 10 min. At suitable intervals, 1 ml. samples were withdrawn for analysis; in all cases a blank determination was made using the same concentration of enzyme in 0.005 M acetate buffer, pH 5.25; some of the substrates (*m*-cresyl, *o*- and *m*-methoxyphenyl, *o*-chlorophenyl and *p*-cyanophenyl β -D-glucosides) contained small amounts of reducing impurity for which the requisite correction was made.

The overall velocity constant, k_0 , was calculated in the usual way from the slope of the linear plot (drawn by the method of least squares) of the decadic logarithm of the concentration of unhydrolysed substrate against the time in minutes. For the determination of K_m , K_a and k'_0 (see below) two or three sets of three or four such determinations of k_0 , using different enzyme concentrations, were carried out with each substrate.

The complete data for the enzymic hydrolyses of phenyl β -D-glucoside are given in Table 3; the full data for the other twenty glucosides are given by Nath (1951).

Acid and alkaline hydrolyses

The hydrolyses were carried out in 50 ml. flasks fitted with cold-finger condensers (Braude, Jones & Stern, 1946) in an oil thermostat at $60^\circ \pm 0.05^\circ$. For the acid hydrolyses the glucosides were taken in 0.2 M concentration in 0.1 N-HCl; for the alkaline hydrolyses 0.01 M solutions in 4 N-NaOH were used. Samples were withdrawn at suitable times, and after neutralization, were analysed by the appropriate method. The first-order velocity constants, k_{acid} and $k_{\text{alk.}}$, were evaluated in the usual manner from the satisfactorily linear log plots and are recorded in Table 4. The values of $k_{\text{alk.}}$ for the *o*- and *p*-nitrophenyl β -D-glucosides were too high for accurate measurement, the former compound being completely hydrolysed in 1 min. and the latter 80% hydrolysed in 3 min.

RESULTS

The results of the experiments on enzymic hydrolysis are expressed in terms of the equilibrium constant, $K_a (=k_1/k_2)$, for the formation of the enzyme-substrate complex and of the first-order velocity constant, k_3 , for the decomposition of this complex according to the well-known Michaelis-Menten representation:

It is more usual to use the Michaelis constant, K_m , which is the dissociation constant for the breakdown of the enzyme-substrate complex into its original components, but we prefer K_a as being more logical, more immediately informative, since it increases with increasing tendency for the overall reaction to proceed from left to right, and more in keeping with standard physico-chemical usage.

Being merely the reciprocal of the Michaelis constant, K_a can be evaluated by any of the methods commonly in use for the evaluation of K_m . We have used the method of Veibel & Lillelund (1940a) (cf. Pigman & Goepp, 1948), in which the reciprocal of the overall velocity constant, k_0 , at a constant enzyme concentration, is plotted against the substrate concentration; the intercept of the resulting straight line on the x axis is equal to $-K_m = -1/K_a$. This method is preferable to other methods (Michaelis & Menten, 1913; Lineweaver & Burk, 1934; Delory & King, 1943) since it employs k_0 , which is accurately determinable, rather than the initial velocity of reaction, which can only be

Fig. 1. Determination of K_a for phenyl β -D-glucoside.

Table 4. *Hydrolysis of substituted phenyl β -D-glucosides*

K_a = Equilibrium constant for hydrolysis by emulsin at 30° in 0.005M acetate buffer, pH 5.25; 1./mole.

k'_s = First-order velocity constant for decomposition of enzyme-substrate complex under above conditions; min.⁻¹.

k_{acid} = First-order velocity constant for hydrolysis at 60° in 0.02M solution in 0.1N-HCl; min.⁻¹.

$k_{alk.}$ = First-order velocity constant for hydrolysis at 60° in 0.01M solution in 4N-NaOH; min.⁻¹.

σ = Substituent constant. (Values for *m*- and *p*-substituents from Hammett, 1940; for *o*-substituents, slightly modified in the light of more recent work, from Mamalis & Rydon, 1950.)

Substituent	K_a			$10^3 k'_s$			$10^4 k_{acid}$	$10^4 k_{alk.}$	σ
	Mean	± S.E.	No. of determinations	Mean	± S.E.	No. of determinations			
H	36.75	± 1.7	3	22.5	± 0.4	10	1.15	2.54	0.000
<i>o</i> -Me	43.4	± 0.5	2	904	± 5	6	1.81	1.16	-0.080
<i>m</i> -Me	52.95	± 4.2	2	56.75	± 1.85	6	1.405	1.92	-0.069
<i>p</i> -Me	76.9	± 0.0	2	17.1	± 0.1	6	2.32	0.960	-0.170
<i>o</i> -Pr ^t	345	± 12	2	56.05	± 1.8	6	—	0.512	—
<i>p</i> -Pr ^t	238	± 56	2	9.11	± 0.39	6	—	0.750	-0.151
<i>o</i> -Bu ^t	132	± 3.5	2	6.675	± 0.22	6	—	0.137	—
<i>p</i> -Bu ^t	157	± 3	2	3.62	± 0.15	6	—	0.672	-0.197
2:4-Me ₂	171	± 11	2	24.9	± 0.3	6	—	0.554	-0.250
2:6-Me ₂	28.8	± 0.2	2	13.3	± 0.3	6	—	1.152	-0.160
<i>o</i> -MeO	58.1	± 0.55	2	965.5	± 10.5	6	8.96	7.62	-0.120
<i>m</i> -MeO	38.5	± 0.75	2	132	± 6	6	0.770	6.28	+0.115
<i>p</i> -MeO	53.45	± 0.85	4	58.8	± 0.95	12	2.407	0.944	-0.268
<i>o</i> -Cl	222	± 22	2	832	± 27.5	6	1.73	230.3	+0.325
<i>m</i> -Cl	102.5	± 2.5	2	228	± 8	6	0.760	19.7	+0.373
<i>p</i> -Cl	119.5	± 5.5	2	44.0	± 0.75	6	0.830	8.96	+0.227
<i>m</i> -CN	182	± 3.5	2	690.5	± 12.5	6	0.509	—	+0.678
<i>p</i> -CN	419.5	± 35	2	286	± 3.5	6	0.366	—	+1.000
<i>o</i> -NO ₂	161.5	± 2.5	2	3515	± 29	6	1.66	> 50 000	+0.650
<i>m</i> -NO ₂	222.5	± 5	2	460	± 13.5	6	0.404	147.4	+0.710
<i>p</i> -NO ₂	500	± 0	2	385	± 2	6	0.330	c. 5000	+1.270

approximated, and does not require the use of such high substrate concentrations. At least two determinations of K_a were carried out, using different enzyme concentrations, with each substrate; our results for phenyl β -D-glucoside (cf. Table 3) are plotted in Fig. 1.

Veibel & Eriksen (1936-7) and Veibel & Lillelund (1940*a*) showed that, under conditions such that the influence of the products on the velocity of the reaction is negligible,

$$k_3 = \frac{k_0(K_m + s_0)}{e_0}, \quad (1)$$

where s_0 and e_0 are the initial molar concentrations of substrate and enzyme, respectively. The value of e_0 , required for substitution in (1) for the evaluation of k_3 , cannot be readily determined since neither the molecular weight of the enzyme nor the purity of the enzyme preparation used are known. We have accordingly replaced e_0 by the weight concentration, e'_0 (mg./l.); the resulting expression

$$k'_3 = k_0 \frac{(K_m + s_0)}{e'_0} \quad (2)$$

gives k'_3 , which is the required first-order constant, k_3 , multiplied by a constant factor (assuming our emulsin preparation to be 1% pure and a molecular weight of 100 000 for pure emulsin, this factor would be 1×10^{-10}). For our present purpose of correla-

tion with substrate structure, k'_3 is just as useful as k_3 . On rearrangement, (2) takes the form (3),

$$\frac{1}{k_0} = \frac{1}{k'_3 e'_0} (K_m + s_0), \quad (3)$$

which is the equation for the linear plot of $1/k_0$ against s_0 , which was used in evaluating K_a ; clearly k'_3 could be determined from the slope of this line but we have preferred the arithmetical method as being more accurate and very nearly as easy.

The values of K_a and k'_3 and of the first-order velocity constants, k_{acid} and $k_{alk.}$, for acid and alkaline hydrolysis of the glucosides investigated by us are given in Table 4.

DISCUSSION

The logarithms of the equilibrium constants, K_a , are plotted against the substituent constants, σ , in Fig. 2. With the exception of the *p*-isopropyl (*p*-Pr^t) and *p*-tert.-butyl (*p*-Bu^t) compounds, the fit is about as good as is usually observed in such correlations. The plot clearly shows that the formation of the enzyme-substrate complex is facilitated by the presence of electron-attracting substituents in the phenyl group. The fact that the points for the *o*-substituents fall satisfactorily on the line indicates that steric effects play a negligible part in the interaction or, at any rate, that part of it directly

influenced by electron-displacement effects; it follows that this interaction must take place in a part of the molecule remote from the phenyl group, namely in the glucose moiety. The most likely form of such interaction subject to polar influences is hydrogen bonding and the facilitating influence of electron-attracting substituents indicates that this must involve the hydroxyl hydrogens of the glucose residue and hydrogen-accepting groups in the enzyme molecule; the alternative possibility of hydrogen bonding between the oxygens of the glucose residue and hydrogens of the enzyme is excluded, since this would be hindered by electron-attracting substituents in the phenyl groups. The observations of Helferich & Lang (1933) and of Pigman & Richtmyer (1942), that certain *O*-methylated glucosides are not hydrolysed by emulsin, are in accordance with the suggested mechanism.

The marked facilitation of enzyme-substrate combination by the bulky alkyl groups, *o*- and *p*-isopropyl and *tert*.-butyl, in spite of their electron-repelling properties, suggests that there is some other type of interaction, probably by means of van der Waals forces, involving the aryl group. That the aglycone half of the molecule must be involved follows from the specificity of the enzyme for β -glucosides and the suggestion that it is attached by van der Waals forces is in accord with

the observation of Veibel & Lillelund (1938) that the equilibrium constant for combination with alkyl β -D-glucosides increases with increasing size of the alkyl group.

The stereochemical specificity of emulsin renders it likely that at least three centres are involved in the attachment of the substrate to the enzyme (Rydon, 1948) and the following partial representation is advanced as at least a plausible working hypothesis:

Fig. 3. Possible mode of attachment of a substituted phenyl β -D-glucoside to emulsin. (The glucopyranose ring lies in the plane of the paper, the benzene ring at right angles to it. —H...X—, hydrogen bond; $\sim$, van der Waals interaction.)

Fig. 2. Correlation of K_a with structure. ●, phenyl β -D-glucoside; ○, *o*-substituted phenyl β -D-glucosides; □, *m*-substituted phenyl β -D-glucosides; ×, *p*-substituted phenyl β -D-glucosides. The line was drawn by the least-square method, neglecting the points for the *o*-substituted compound and for the *p*-isopropyl and *p*-*tert*.-butyl compounds.

In the figure, the two hydrogen-bonds leading to the glucose residue are intended to represent, but not specify, at least two bonds of this type between the enzyme and the glucopyranose ring.

The logarithms of the first-order velocity constants, k_{alk} and k_{acid} , for the alkali- and acid-catalysed hydrolyses are plotted against σ in Figs. 4 and 5. The effect of substitution on the two reactions is that expected on general grounds, the acid-catalysed reaction, which depends on attack by the electrophilic proton, being facilitated by electron-repelling substituents, and the alkali-catalysed reaction, in which the attack is by the nucleophilic hydroxyl ion, being facilitated by electron-attracting groups. The reaction constants, ρ (Hammett, 1938, 1940) for the two reactions, +2.48 and -0.66, respectively, are normal for the two types of reaction (cf. Taft, 1952); Dyfverman & Lindberg (1950) found $\rho = 2.5$ in an investigation of the alkaline hydrolysis of a less extensive series of substituted phenyl β -D-glucosides. Both reactions show facilitation by *o*-nitro, *o*-chloro and *o*-methoxy groups. Facilitation of alkaline hydrolysis by an *o*-nitro group is very pronounced and has been noted by previous workers (Montgomery, Richtmyer & Hudson, 1943; Snyder & Link, 1952); it is readily explicable on the basis of hydrogen bonding with the 2-hydroxyl group of the glucose residue,

which leads to enhanced electrophilic reactivity of the relevant carbon atom in the phenyl group, thus:

and the weaker facilitation by *o*-chloro and *o*-methoxy groups may have a similar explanation. The effect of these three substituents on the acid-catalysed hydrolysis is not readily explicable. The bulky *isopropyl* and *tert.*-butyl groups, when present as *o*-substituents, cause marked inhibition of alkaline hydrolysis, no doubt owing to conventional steric hindrance.

Fig. 6 shows $\log k'_2$ plotted against σ . It is at once apparent that the decomposition of the enzyme-substrate complex, although it occurs in acid solution, resembles alkaline, rather than acid, hydrolysis (contrast Pigman & Goepp, 1948) in being facilitated by electron-attracting substituents. Clearly, the enzyme-substrate complex decomposes under the attack of some nucleophilic reagent; whether this is free hydroxyl ion, adsorbed hydroxyl ion or

hydroxyl ion arising from hydrated amino-groups, is a question which cannot profitably be discussed on the basis of the available evidence.

Fig. 5. Correlation of k_{acid} with structure. For key, see Fig. 2. The line was drawn by the least-square method, neglecting the points for the *o*-substituted compound.

Fig. 4. Correlation of $k_{\text{alk.}}$ with structure. For key, see Fig. 2. The line was drawn by the least-square method, neglecting the points for the *o*-substituted compounds.

Fig. 6. Correlation of k'_2 with structure. For key, see Fig. 2. The lines were drawn by the least-square method, the upper one using the points for the *m*-substituted compounds and the lower one using the points for the unsubstituted and *p*-substituted compounds.

It is also clear from Fig. 6 that marked steric influences are superimposed on the more usual electronic influences. The points for the *p*-substituted compounds fall reasonably well on a straight line passing through the point for the unsubstituted compound, with the exception of those for the *p*-isopropyl and *p*-*tert*-butyl compounds; the sluggish decomposition of these two complexes is very probably due to the strength of the van der Waals component of the forces holding them together, and it may well be that it is the enzyme-product complex which is unusually stable in these cases. The *m*-substituted compounds also fall well on a straight line which is, however, considerably displaced towards higher k'_3 values from the point for the unsubstituted compound. The *o*-substituted compounds, again with the exception of the isopropyl and *tert*-butyl compounds, which, like their *p*-isomerides, may owe their stability to strong van der Waals interaction, and also with the curious exception of the dimethyl compounds, show very markedly enhanced instability; the ratios of the values of k'_3 for the *o*- and *p*-isomerides are as follows:

Substituent	Me	Cl	MeO	NO ₂	Pr ⁱ	Bu ^t
k'_3 for <i>o</i> -/ k'_3 for <i>p</i> -	52.8	18.9	16.3	9.1	6.2	1.8

Clearly the effect here, and also in the *m*-series, is due to the size, rather than the electronic properties, of the substituent, and it is tempting to think that what is involved is a tilting of the phenyl group in Fig. 3, aided by the close fitting of the substituent into a recess in the enzyme of about the size and shape of a methyl group, which results in the key carbon atom of the phenyl group becoming more accessible to attack by the nucleophilic entity involved; more experimental evidence is, however, required before this matter can be pursued further.

SUMMARY

1. The hydrolysis by emulsin of phenyl β -D-glucoside and twenty 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. First-order velocity constants for the alkaline and acid hydrolysis of most of the glucosides have also been determined.

3. The results have been correlated with the electronic properties of the substituents, as measured by their substituent constants; acid hydrolysis is facilitated by electron-repelling substituents but alkaline hydrolysis and the formation and breakdown of the enzyme-substrate complex are all facilitated by electron-attracting substituents.

4. A mechanism is suggested for the combination of emulsin with aryl β -D-glucosides.

5. The mechanism of the decomposition of the enzyme-substrate complex is discussed; in this reaction steric effects, as well as electronic effects, appear to play an important part.

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The Need for Selenite and Molybdate in the Formation of Formic Dehydrogenase by Members of the *Coli-aerogenes* Group of Bacteria

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Factors required in trace amounts for growth are generally considered to play a catalytic role in the metabolism of the organism, and comparison of the enzymic activity of normal and deficient cells has yielded information concerning the function of organic growth factors in the metabolism of micro-organisms. Waring & Werkman (1944) have used this method to study the function of iron in *Aerobacter indologenes*; they found that the activity of formic dehydrogenase, among other enzymes, was greatly reduced in iron-deficient cells. It does not follow, however, that only such inorganic factors as are required for normal growth are concerned in the formation of enzymes, since an enzyme whose function is not indispensable to the anabolism of the cell could be greatly reduced in amount without any effect on growth being apparent. This paper describes an instance of this sort; traces of selenite and molybdate, as well as iron, were found to be essential for the formation of an active formic dehydrogenase by strains of coliform organisms grown in a purified synthetic medium, although the first two factors, i.e. selenite and molybdate, had no influence on growth.

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EXPERIMENTAL

Organisms. That most commonly used was 1433, a strain of *Escherichia coli* obtained from Dr M. R. Pollock. Essentially similar results were obtained using two other strains, N.C.T.C. no. 86, and *Esch. coli* S from the collection of Dr C. B. van Niel, and two strains of *Aerobacter aerogenes*, N.C.T.C. no. 5936, and *Aero. aerogenes* 1, supplied by Dr E. F. Gale.

Purification of chemicals. Since conflicting results were obtained in the early experiments, in which the 8-hydroxyquinoline extraction technique described by Waring & Werkman (1944) was used, a more rigorous method of purification was resorted to. The individual chemicals employed in the preparation of the media were treated as described below. The techniques were designed so as to eliminate traces of impurities by distillation where possible, and also to make use of the capacity of $\text{Fe}(\text{OH})_3$ to adsorb traces of selenite among other ions.

Certain general precautions were taken in all manipulations. Only Pyrex glassware was used; each item carefully cleaned immediately before use by rinsing first with ethanolic KOH, followed by distilled water, conc. HCl, and finally with glass-distilled water. Care was taken throughout to keep beakers and the necks of flasks covered with hardened filter paper or with cleaned beakers to avoid contamination with dust. The water and solvents used during the purifications were freshly distilled in an all-Pyrex still.

The purified chemicals were stored in cleaned, wide-mouthed Pyrex bottles, and kept in a clean desiccator to