2. After a preliminary incubation period of 45 min., i.e. after a steady state had been established, ^{42}K in the form of ^{42}KCl was added to slices of guinea pig-brain cortex and to pieces of ox retina, and the rate of migration of ^{42}K into the tissue was measured. From these measurements the rate of potassium exchange in the steady state was calculated. The turnover rate $\left(\frac{\text{potassium exchanged/min.}}{\text{potassium present in tissue}} \times 100 \right)$, was 3.5–4 in brain cortex and 7–10 in retina.

3. With liver (guinea pig, pigeon), pancreas (pigeon) and smooth muscle (pigeon gizzard) the normal potassium gradient between tissue and environment was not maintained when the tissues

were suspended in saline media or homologous serum. The bearing of this observation on the finding of Buchanan *et al.* (1949) that the rate of synthesis of glycogen in liver slices is optimal when the potassium concentration of the medium resembles that of the tissue is discussed.

4. The potassium level of sliced guinea pig-kidney cortex behaved similarly to that of brain and retina except that α -ketoglutarate was more effective in maintaining the normal level than L-glutamate.

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Studies in Detoxication

35. SPECTROPHOTOMETRIC DETERMINATION OF β -GLUCURONIDASE USING *p*-CHLOROPHENYLGLUCURONIDE AS SUBSTRATE

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In recent years, there has been a considerable interest in the enzyme β -glucuronidase. Its only proved activity is the hydrolysis of conjugated β -glucuronides, but the work of Levvy and his co-workers (Levy, Kerr & Campbell, 1948; Karunairatnam, Kerr & Levvy, 1949*a*; Kerr, Campbell & Levvy, 1949, 1950; see also Odell & Fishman, 1950) shows that when there is an increase in proliferative

activity in certain tissues there is also an increase in the β -glucuronidase activity. The enzyme has been found to occur in increased amounts in cancer tissue (Fishman & Anlyan, 1947; Karunairatnam, Kerr & Levvy, 1949*b*; Odell & Burt, 1949) and in tissues undergoing repair after damage (Levy *et al.* 1948). The use of β -glucuronidase as an agent for the smooth hydrolysis of β -glucuronides is likely to increase,

because hydrolysis by acid often leads to low yields of the aglycone. There is little doubt that several β -glucuronidases occur in nature, for glucuronidase activity has been detected in a variety of animal tissues, in plants (e.g. the baicalinase of Miwa (1932, 1934-5, 1936-7) appears to be a β -glucuronidase), in bacteria (Buehler, Katzman, Doisy & Doisy, 1949; Barber, Brooksbank & Haslewood, 1948) and in moulds (Spencer & Williams, 1950*a*).

The assay of β -glucuronidase can be carried out by a determination of either the glucuronic acid or the aglycone set free during its hydrolytic action on conjugated glucuronide. Most earlier methods depended on the non-specific reducing properties of glucuronic acid, but more recent methods rely on the estimation of the freed aglycone, and the monoglucuronides of oestriol, phenolphthalein and phenol have been used for this purpose. The use of oestriol monoglucuronide (Fishman, 1939) as a substrate is limited by its relative inaccessibility. Phenylglucuronide, however, is easily obtained (Garton, Robinson & Williams, 1949), but the estimation of phenol by the Folin-Ciocalteu reagent is subject to variable blanks due to protein (Kerr, Graham & Levvy, 1948). Phenolphthalein monoglucuronide has not been obtained crystalline and is only available as a cinchonidine salt after a somewhat tedious isolation procedure (Di Somma, 1940; Talalay, Fishman & Huggins, 1946; Fishman & Talalay, 1947; Fishman, Springer & Brunetti, 1948). Nevertheless, the use of phenolphthalein glucuronide appears to us to be one of the best methods for the assay of β -glucuronidase.

The present method arose as a result of two observations, the one being the ease with which pure crystalline *p*-chlorophenylglucuronide could be prepared and the other, the sharp differences in ultraviolet absorption spectra between the anion and the glucuronide of *p*-chlorophenol. The principle involved in the present determination of *p*-chlorophenol in the presence of its glucuronide is a general one and is applicable to the determination of a number of hydrolytic enzymes where the substrate, whose absorption spectrum is practically the same in acid and alkaline solution, contains a phenol whose spectrum in alkali is different from that in acid. The same principle is involved in the phenolphthalein glucuronide method for the assay of β -glucuronidase, but in this case the colour measured is in the visible part of the spectrum. A method of the same type is already being applied in this laboratory to sulphatase determinations using *p*-chloro- and *p*-nitro-phenylsulphuric acids as substrates (Robinson, Spencer & Williams, 1951).

EXPERIMENTAL

Preparation of p-chlorophenylglucuronide. The preparation of this compound, described by Spencer & Williams

(1950*b*), has been improved as follows. A total of 6 g. of *p*-chlorophenol (m.p. 42.5°) was fed as a warm water emulsion to four rabbits. The 12 hr. urine (250 ml.) was filtered through glass wool, made 0.2*N* with respect to HCl and then continuously extracted with ether for 16 hr. The glucuronide crystallized as plates in the extract, and after cooling to 0° was filtered off. Concentration of the filtrate yielded further crystals. The combined solids (6.7 g.) were recrystallized twice from small volumes of hot water with the addition of charcoal. The monohydrate of the glucuronide was thus obtained pure as long colourless plates (yield 5.2 g.), m.p. 151° and $[\alpha]_D^{25} - 82^\circ$ (c, 0.5 in water).

Fig. 1. Ultraviolet absorption spectra of *p*-chlorophenol in 0.1*N*-NaOH (—) and in 0.1*N*-HCl (---), and of *p*-chlorophenylglucuronide in 0.1*N*-NaOH (— · —).

Table 1. Absorption spectra of *p*-chlorophenol and its glucuronide

Solvent	<i>p</i> -Chlorophenylglucuronide monohydrate		<i>p</i> -Chlorophenol	
	$\lambda_{\max}$ (m μ)	$\epsilon_{\max}$	$\lambda_{\max}$ (m μ)	$\epsilon_{\max}$
Water	219.5	10,700	—	—
	275.5	930	—	—
0.1 <i>N</i> -HCl	220	10,700	222	8,900
	275.5	910	280	1,600
0.1 <i>N</i> -NaOH	219.5	10,800	245	11,650
	276	970	298	2,600

Absorption spectra. All absorption spectra were determined with a Unicam spectrophotometer, Model S.P. 500. The ultraviolet absorption spectra of *p*-chlorophenylglucuronide in 0.1*N*-HCl and 0.1*N*-NaOH are identical (Spencer & Williams, 1950*b*), but *p*-chlorophenol itself exhibits different spectra in these solvents (see Smith, Spencer & Williams, 1950). The relevant data have been redetermined and are given in Table 1 and Fig. 1. It is clear from these data that the absorption maxima of *p*-chlorophenol in 0.1*N*-NaOH occur at wavelengths where the absorption of the glucuronide is minimal. Thus by measuring the increase of absorption in 0.1*N*-NaOH at 245 or 298 m μ . of a *p*-chlorophenylglucuronide solution under

going hydrolysis, the amount of *p*-chlorophenol formed can readily be calculated. It can be assumed for practical purposes that all the change in absorption at these wavelengths is due to the formation of *p*-chlorophenol since the error introduced by neglecting the decrease in absorption due to hydrolysis of the glucuronide is very small (see Table 2).

Table 2. *Molecular extinctions of p-chlorophenol and its glucuronide at 245 and 298 m μ . in 0.1N-NaOH*

(100. *B/A* is the percentage error introduced by neglecting decrease in absorption due to hydrolysis of glucuronide.)

λ (m μ .)	Extinction (ϵ)		100. <i>B/A</i>
	<i>p</i> -Chlorophenol <i>A</i>	<i>p</i> -Chloro-phenyl-glucuronide <i>B</i>	
245	11,600	60	0.5
298	2,600	5	0.2

The data presented in Fig. 2 show that the maxima of *p*-chlorophenol in 0.1N-NaOH obey the Lambert-Beer law. The effect of the acetate buffer and ethanol in the concentrations used in the glucuronidase assay were also investigated. It was found that these substances had no effect on the extinction coefficients of the phenol when due allowance for them was made in the blank cell of the spectrophotometer.

Fig. 2. Relation between the extinctions (*E*) at 245 and 298 m μ . and the concentration of the *p*-chlorophenol anion, demonstrating that the Lambert-Beer Law is obeyed.

Preparation of the glucuronidase extract. The extract used was obtained from ox spleen by a method based on the work of Talalay *et al.* (1946), Graham (1946) and Mills (1948). All pH measurements were made with the Cambridge pH-meter.

A freshly removed ox spleen was stripped of fat and the tissue (800 g.) homogenized with chilled water in an Atomix homogenizer. The temperature did not rise above 15°. The homogenate was centrifuged and the aqueous portion adjusted to pH 5 with *N*-acetic acid, and 25 ml. *M*-acetate buffer (pH 5) added to stabilize the pH. The mixture was then incubated for 1 hr. at 38°. The flocculated proteins were then centrifuged and the dark-red supernatant fluid (500 ml.) was half saturated with (NH₄)₂SO₄ and kept overnight at 0°. The precipitate which formed was collected on the centrifuge, dissolved in 200 ml. water and dialysed against running water for 24 hr. Further purification was effected by the method of Mills (1948) by precipitation with (NH₄)₂SO₄ between the limits of 1.25–1.54 *M* and 1.54–1.75 *M*, whereby two fractions (I and II) were obtained. Each fraction was twice reprecipitated between its limits and was stored at 0°. No deterioration in activity was observed during 3 weeks at this temperature.

Estimation of glucuronidase. For the glucuronidase estimations a mixture of the enzyme extract, substrate and buffer was incubated. The reaction was then stopped at the appropriate time and the liberated phenol estimated spectrophotometrically.

The most suitable buffer for the active pH range of the enzyme is 0.2M-acetate buffer because its light absorption in the regions of the spectrum involved here is very small, and it does not inhibit the enzyme (Mills & Paul, 1949). The protein present in the enzyme extracts, however, has an appreciable absorption in alkaline solution at 245 and 298 m μ . and must be removed before the absorption due to *p*-chlorophenol can be measured. It was found that the protein could be completely precipitated and the reaction stopped by the addition of absolute ethanol to a final concentration of 80% (v/v). After removal of the protein by centrifugation, a portion of the solution was made 0.1N to NaOH and the absorption measured in the spectrophotometer. No hydrolysis of the glucuronide solution occurred on incubation for 1–3 hr. with buffers at pH's between 5.6 and 3.75 and hence no blank of 'substrate incubated with buffer' was used in the estimation. Phenolic glucuronides as a rule required strong acids for hydrolysis and are stable to alkali. The final procedure adopted was as follows:

A series of four 15 ml. centrifuge tubes were set up. To two of these tubes (Tests) there were added, in order, 0.4 ml. each of 0.2M-acetate buffer, *p*-chlorophenylglucuronide solution and enzyme extract. The solutions were mixed by rotating. All three solutions had been adjusted to the required pH and temperature of incubation before mixing. To the other two tubes (Controls) there were added 0.4 ml. each of 0.2M-acetate buffer and enzyme extract adjusted to the required pH and temperature. All four tubes were then incubated at 37°. After the desired period of incubation, 0.4 ml. of the adjusted glucuronide solution was added to each of the control tubes. Then 4.8 ml. of absolute ethanol was added to each of the four tubes to stop the reaction and precipitate protein. The tubes were centrifuged for 3 min. to remove the protein. Then 5 ml. of the clear supernatant fluid were transferred from each tube to 10 ml. standard flasks, and made up to the mark with 0.2N-NaOH. The extinctions of these solutions at 245 m μ . (1 mm. slit) were read in the spectrophotometer against a blank solution prepared as in the case of the test solutions except that 0.8 ml. of water had been used instead of the 0.4 ml. of the glucuronide solution and 0.4 ml. enzyme extract. In those instances where the β -glucuronidase activity was not known

approximately, and proved to be so great as to bring the extinction reading beyond the accurate portion of the spectrophotometer scale, accurate readings could be obtained by measuring the absorption at 298 m μ . (with 0.5 mm. slit) instead of at 245 m μ . Generally, however, with very active solutions of β -glucuronidase, estimations should be repeated by diluting the enzyme solution so that between 10 and 40 μ g. of *p*-chlorophenol were liberated.

For readings taken at 245 m μ ., the amount of *p*-chlorophenol liberated is given by the following equation:

$$\mu\text{g. } p\text{-chlorophenol} = \frac{(E_t - E_c) \times 128.5 \times 6 \times 10,000}{11,650 \times 5} = 132.3(E_t - E_c),$$

where, E_t and E_c are the observed extinctions of the test and control solutions respectively; 128.5 = mol. wt. of *p*-chlorophenol; 11,650 = molecular extinction of the *p*-chlorophenol anion at 245 m μ .

For 4 μ g. *p*-chlorophenol, the value of $E_t - E_c$ is 0.03 which is easily read on the spectrophotometer.

The recovery of p-chlorophenol. Since the procedure described above involves the precipitation of protein, it was important to show that *p*-chlorophenol was not lost by adsorption on the protein. The recovery of added *p*-chlorophenol was tested by carrying out the procedure described for the test solutions in the preceding section, but replacing the glucuronide substrate by standard solutions of *p*-chlorophenol. The recovery of the phenol was tested with and without incubation and proved to be $100 \pm 4\%$

Table 3. *Recovery of p-chlorophenol in glucuronidase assay method*

(Method see text, p. 539; spectrophotometric determination of anion at 245 and 298 m μ .)

Added (μ g.)	<i>p</i> -Chlorophenol	
	Recovered	
	Without incubation (%)	With incubation (%)
Measured at 245 m μ .		
8.42	103	103.5
16.84	102.5	99
25.26	98	103
33.68	101.5	96.5
42.10	100	98
58.94	102	100.5
Measured at 298 m μ .		
106.3	97	99
212.6	100	98.5
318.9	102	100.5
425.2	102.5	102

for the ranges 8–425 μ g. (Table 3). No chlorophenol was lost on the protein precipitate. Further experiments showed that small amounts of added *p*-chlorophenol could be recovered within $\pm 1\%$ in the presence of relatively large amounts of the substrate.

The effect of pH on the activity of the enzyme

The effect of pH on the β -glucuronidase activity of the fractions I and II in 0.2M-acetate buffer was investigated using a substrate concentration of 0.015M and incubating at 37° for 1 hr. The *p*-chloro-

phenylglucuronide solution was brought to the desired pH electrometrically by titration with 0.2N-NaOH. The pH's of all solutions were checked with the Cambridge pH-meter. The pH-activity curves of fractions I and II were practically identical and the curve for fraction II is given in Fig. 3. Two maxima

Fig. 3. pH-activity curve of fraction II (see text, p. 539) of an ox-spleen β -glucuronidase preparation. Activity expressed as percentage of maximum.

were found, one at pH 4.1 and the other at 5.2, the extracts showing greatest activity at the latter pH. Mills (1948) has reported the separation from ox spleen of two fractions showing β -glucuronidase activity with pH optima at 4.5 and 5.2, using *laevo*-menthyl, phenyl and phenolphthalein glucuronides as substrates. Further work by Mills and his co-workers (Mills & Paul, 1949; Mills, Paul & Smith, 1949) has demonstrated the presence of ox spleen of a third glucuronidase with a pH optimum of 3.4 acting only upon phenyl- β -glucuronide. Since the work reported in the present paper was done, reports have appeared in the literature (Sarkar & Sumner, 1950; Bernfeld & Fishman, 1950) which suggest that there is only one glucuronidase with an optimum pH at about 4.5. Although a pure enzyme preparation has not been used in this work the Lineweaver & Burk analysis below suggests that we may have been dealing with a single enzyme. It should be emphasized, however, that we are more concerned in this paper with the application of the spectrophotometric method than with enzyme kinetics.

The effect of substrate concentration and calculation of the Michaelis constant

The effect of varying the substrate concentration on the glucuronidase activity of fraction II at pH 5.2 and at 37° using an incubation period of

1 hr. is shown in Fig. 4. Optimal rate ($V_{\text{opt.}}$) was observed when the *p*-chlorophenylglucuronide concentration was $2.85 \times 10^{-2} \text{ M}$ ($S_{\text{opt.}}$) and further increases in substrate concentration caused marked inhibition of the enzyme. Inhibition of glucuronidase activity by excess substrate has been previously observed by Talalay *et al.* (1946) with phenolphthalein glucuronide and by Kerr *et al.* (1948) with

Fig. 4. Relation between substrate (*p*-chlorophenylglucuronide) concentration and β -glucuronidase activity.

phenylglucuronide, but neither group of workers analysed their data with a view to ascertaining the inhibitory mechanism involved. We have, however, been able to apply the tests proposed by Lineweaver & Burk (1934) to our data and have concluded that the mechanism involved is a pure substrate inhibition.

The reactions involved in substrate inhibition are

where E = enzyme, S = substrate, ES is the active and ES_n is the inactive enzyme-substrate complex. The equation for general substrate inhibition derived by Lineweaver & Burk (1934) on the Michaelis-Menten treatment is

$$(S)/v = (K_m + (S) + (S)^n/K_2)/V_{\text{max.}}, \quad (1)$$

where (S) = substrate concentration; v = observed velocity; K_m = dissociation constant of the ES complex, i.e. the Michaelis constant; K_2 = dissociation constant of ES_n complex; $V_{\text{max.}}$ = ideal maximal velocity of the enzyme reaction if there were no inhibition by excess substrate; n = number of molecules of substrate combined with enzyme-substrate complex.

The plot of $[S]/v$ against $[S]$ gave a concave curve which was linear at low values of $[S]$ (Fig. 5). From the ratio of the ordinate intercept ($K_m/V_{\text{max.}}$) and the slope ($1/V_{\text{max.}}$) of the linear portion of the curve

where substrate inhibition is negligible, the Michaelis constant K_m was calculated to be $3.0 \times 10^{-2} \text{ M}$.

The values derived from Fig. 5 were then used to plot $\log ([S]/v - K_m/V_{\text{max.}} - [S]/V_{\text{max.}})$ against $\log [S]$, the graph of which is given in Fig. 6. From this figure the value of n , which is the slope drawn to the nearest whole number (Lineweaver & Burk, 1934), can be determined. In fact we found $n = 3.95$ and, making $n = 4$, the value of K_2 , the dissociation constant of the inactive complex, was calculated to be $6.7 \times 10^{-5} \text{ M}$.

Fig. 5. Plot of substrate concentration $[S]$ against $[S]/v$; see text, p. 541. (v is expressed in $\mu\text{g. p-chlorophenol}$ liberated/ml. of enzyme solution.)

Fig. 6. Plot of $\log [S]$ against $\log ([S]/v - K_m/V_{\text{max.}} - [S]/V_{\text{max.}})$.

From the slope the value of n is calculated (see text, p. 541).

If the mechanism proposed is the true one, i.e. it is case III of Lineweaver & Burk (1934) then $[S]/v$ plotted against $([S] + [S]^n/K_2)$ should give a straight line whose slope is $1/V_{\text{max.}}$ and ordinate intercept is $K_m/V_{\text{max.}}$. Fig. 7 shows that this plot is a straight line and furnishes good evidence that the proposed reactions are the ones operating. The data did not

conform with any of the other six cases analysed by Lineweaver & Burk. It will be shown later that sodium glucuronate is an inhibitor of glucuronidase, but in the concentrations liberated in this experiment its effect is negligible.

Fig. 7. Plot of $[S]/v$ against $([S] + [S]^n/K_2)$. The straight line shows that the proposed mechanism of competitive substrate inhibition is the true one (see text, p. 541).

Fig. 8. Graph showing the relative distribution of E, ES (active) and ES_n (inactive) as a function of substrate concentration. E represents the total concentration of enzyme free and combined; ES (no inhibition), the calculated concentration of active enzyme-substrate complex if there were no inhibition; ES_n , the calculated concentration of inactive enzyme-substrate complex; ES (inhibition) represents the calculated concentration of active enzyme-substrate complex when inhibition occurs. The points on the curve ES (inhibition) are experimental values (see Fig. 4).

From the values of K_m , K_2 , and V_{max} obtained in the above analysis, the relative distribution of E, ES (active) and ES_n (inactive) has been calculated and is presented in Fig. 8. It will be noted that there

is considerable agreement between the experimental results and the calculated curve for ES in the presence of ES_n .

The values of the various constants are given in Table 4.

Table 4. Values of various constants for the action of β -glucuronidase on *p*-chlorophenylglucuronide at pH 5.2

V_{opt} = optimum velocity; relative values taking $V_{max} = 100$. The calculated value is obtained from the equation

$$V_{opt} = \frac{(n-1)V_{max}}{(n-1) + nK_m \sqrt[n]{\frac{(n-1)}{K_m K_2}}}$$

The calculated value for $[S]$ at $V_{opt} = \{K_m K_2 / (n-1)\}^{1/n}$.

Constant	Value	
	Calculated	Observed (Fig. 4)
K_m	$3 \times 10^{-2} M$	—
K_2	—	$0.82 \times 10^{-2} M$
V_{opt}	41.7	42
$[S]$ at V_{opt}	$2.87 \times 10^{-2} M$	$2.85 \times 10^{-2} M$
K_2	$6.7 \times 10^{-5} M$	—
n	4	—

Effect of time. Using extract II the effect of varying the incubation period on the release of *p*-chlorophenol by the enzyme was studied at pH 5.2 and 37° and a

Fig. 9. Relationship of enzyme activity to time (0.4 ml. of suitably diluted extract II at pH 5.2 and 37°, with $1.5 \times 10^{-2} M$ *p*-chlorophenylglucuronide).

straight-line relationship was observed (Fig. 9) over a period of 3.5 hr. The maximum concentration of glucuronic acid liberated during this time was $2.1 \times 10^{-4} M$, an amount which is not inhibitory (see p. 544). Similarly, *p*-chlorophenol was not inhibitory in the concentrations liberated in these experiments.

Effect of enzyme concentration. The results are shown in Fig. 10, which shows a straight-line relationship between the amount of enzyme and *p*-chlorophenol liberated up to 50 μ g.

Fig. 10. Plot of enzyme activity (expressed in μ g. *p*-chlorophenol liberated) against relative enzyme concentration (in arbitrary units). Conditions as for final assay procedure, p. 543. Extract II, pH 5.2, 37°; time of incubation, 1 hr.; substrate concn., 1.5×10^{-2} M.

Final assay procedure

From the results of the above experiments it was concluded that optimal β -glucuronidase activity occurred at a substrate concentration of 2.85×10^{-2} M after a 1 hr. incubation at 37° and pH 5.2. Various considerations, which include conservation of substrate, the value of control readings and activity, lead us to recommend a substrate concentration of 1.5×10^{-2} M for general assay work. At this concentration, activity of the enzyme is 80 % of the maximum value and the control reading, due to the absorption of the substrate which remains constant, is about 0.250, bringing the test readings into the most accurate portion of the spectrophotometer scale. For convenience and accuracy the enzyme solutions used should be diluted so that they liberate between 10 and 40 μ g. *p*-chlorophenol.

The assay procedure is the same as detailed on p. 539, using 1.5×10^{-2} M-substrate, all solutions being adjusted to pH 5.2 and 37° before mixing. An incubation period of 1 hr. is used. Under these conditions the amount of *p*-chlorophenol liberated is proportional to the enzyme present (see Figs. 9, 10).

Unit. A *p*-chlorophenyl unit of β -glucuronidase activity may be defined as the amount of enzyme which at pH 5.2 and 37° in 1.5×10^{-2} M-*p*-chlorophenylglucuronide and 0.2 M-acetate buffer liberates 1 μ g. of *p*-chlorophenol in 1 hr.

The effect of inhibitors on β -glucuronidase

The inhibition of β -glucuronidase by various compounds has been investigated by Karunairatnam & Levvy (1949) who examined the inhibitory effects of certain sugar acids and a variety of miscellaneous substances. The most potent inhibitor was D-glucosaccharic acid. These results were largely confirmed by Mills & Paul (1949), who in a brief communication reported inhibition by some dibasic acids and monobasic and dibasic polyhydroxy acids which were not named in their communication. Our main interest was the application of the spectrophotometric method to the study of β -glucuronidase, but in view of the differences in structure between D-glucurone and D-glucuronic acid it was interesting to apply our method to the study of the inhibition of glucuronidase by these substances.

Materials

D-Glucurone, m.p. 171–173°, $[\alpha]_D^{25} + 19.85^\circ$ (c, 1.76 in water) was the gift of Corn Products Refining Co.

Sodium D-glucuronate monohydrate was prepared by adding exactly one equivalent of 0.1 N-NaOH to a solution of D-glucurone. On mixing the two substances, the mixture turned yellow, but the colour faded in 10 min. After keeping overnight the solution was concentrated to a small volume *in vacuo* and the Na salt precipitated by addition of ethanol. A repetition of the precipitation process gave the salt as a white amorphous powder with $[\alpha]_D^{25} + 22.5^\circ$ (c, 0.8 in water). (Found: Na, 10.0; H₂O, 7.5. Calc. for C₆H₆O₇Na.H₂O, Na, 9.8; H₂O, 7.7 %.) Ehrlich & Rehorst (1929) record $[\alpha]_D + 22.5^\circ$ for sodium D-glucuronate monohydrate after mutarotation.

Sodium mucate was prepared by addition of the equivalent of 0.1 N-NaOH to mucic acid, m.p. 222° (decomp.).

Sodium potassium D-glucosaccharate was similarly prepared from acid potassium saccharate.

Methods

The determinations were carried out as described for the estimation of glucuronidase (p. 543), using 0.2 ml. of inhibitor solution and 0.2 ml. *p*-chlorophenylglucuronide solution instead of the 0.4 ml. of glucuronide solution in both the test and control tubes. For comparison of the rate in the absence of the inhibitor, 0.2 ml. water was substituted for the inhibitor solution. All solutions were previously adjusted to pH 5.2.

Calculation of K_I , the dissociation constant of enzyme-inhibitor complex

Competitive inhibition can be represented by the reaction

where E = enzyme, S = substrate and I = inhibitor. By the treatment of Michaelis & Menten (1913), Lineweaver & Burk (1934) derive the equation

$$\frac{1}{v} = \frac{1}{V_{\max.}} \left(K_m + \frac{K_m[I]}{K_I} \right) \frac{1}{[S]} + \frac{1}{V_{\max.}}, \quad (2)$$

where $[I]$ is the concentration of inhibitor and K_I is the dissociation constant of the enzyme-inhibitor complex EI. Other symbols are as defined on p. 541.

Lineweaver & Burk utilize the above equation by plotting $1/v$ against $1/[S]$ and competitive inhibition is shown by an increase in $(\text{slope} \times V_{\max.})$, K_I being calculated from the values of the slope and intercept. However, in the present study there is the added complication of inhibition by excess substrate. This difficulty can be avoided by working at substrate concentrations where substrate inhibition is negligible, but this restricts the range of concentrations to 0–0.01 M (see Fig. 8), where glucuronidase activity is low. We have therefore overcome the difficulty by fixing the substrate concentration at a position where glucuronidase activity is highest without substrate inhibition being of importance (0.01 M) and then varying the concentration of the inhibitor. To suit our requirements the above equation (2) can be rearranged in the form $y = mx + c$ as follows:

$$\left(\frac{V_{\max.}}{v} - 1\right) = \frac{K_m}{K_I[S]} [I] + \frac{K_m}{[S]}. \quad (3)$$

If the inhibition is competitive the plot of $(V_{\max.}/v) - 1$ against $[I]$ should produce a straight line whose ordinate intercept is $K_m/[S]$ and slope

Table 5. Dissociation constants of β -glucuronidase-inhibitor complexes

Inhibitory	K_I (10^{-3} M)	Affinity ($1/K_I$)
Sodium potassium D-glucosaccharate	0.88	1136
Sodium D-glucuronate	11.1	90
Sodium mucate	36.2	28
D-Glucurone	Non-inhibitory	—

Fig. 11. Graph showing that the inhibition of β -glucuronidase by D-glucosaccharate is competitive (see text, p. 544).

$K_m/K_I[S]$, so that K_I is given by the ratio (intercept:slope). K_m and $V_{\max.}$ for *p*-chlorophenylglucuronide are already known (see Table 4). The results of this method of analysis show that all the

inhibitory compounds listed in Table 5 inhibit competitively, because, as shown for D-glucosaccharate in Fig. 11, the plot of $(V_{\max.}/v) - 1$ against $[I]$ is a straight line and the ordinate intercept is the calculated value of $K_m/[S] = 3$.

DISCUSSION

The procedure presented in this paper provides a convenient method for the assay of β -glucuronidase. Although no claim is made that the present procedure is superior to that using phenolphthalein glucuronide, it possesses considerable advantage in the case of preparation of the substrate, *p*-chlorophenylglucuronide. Using this compound, optimal β -glucuronidase activity was found at a substrate concentration of 2.85×10^{-2} M when measured at pH 5.2 and incubated at 37° for 1 hr.

Applying the spectrophotometric method, the kinetics of the enzymic reaction have been studied and the inhibition occurring at high substrate concentration has been shown, following the analytical procedure of Lineweaver & Burk (1934), to be probably due to the formation of an inactive enzyme-substrate compound of suggested formula ES_4 . The analysis has also provided a value of the Michaelis-Menten constant ($K_m = 3 \times 10^{-2}$ M). The recorded Michaelis constants for β -glucuronidase acting on various substrates are given in Table 6.

Table 6. Michaelis constants (K_m) of β -glucuronidase

Substrate	K_m (M)	pH	Reference
Phenolphthalein monoglucuronide	0.00005	4.5	Talalay <i>et al.</i> (1946)
<i>laevo</i> -Menthylglucuronide	0.004	5.3	Fishman (1939)
Oestriol monoglucuronide	0.005	4.3	
<i>laevo</i> -Bornylglucuronide	0.01	4.4	
Phenylglucuronide	0.0035*	5.2	Kerr <i>et al.</i> (1948)
<i>p</i> -Chlorophenylglucuronide	0.03	5.2	This paper

* $K_{1/2}$ in this instance (see text).

All the values of K_m quoted in this table except that of phenylglucuronide have been calculated according to Lineweaver & Burk (1934). The substrate concentration-activity curve of phenylglucuronide does not lend itself to calculation of K_m by this method, and the figure quoted by Kerr *et al.* (1948) is actually $K_{1/2}$. Since excess substrate inhibition by this glucuronide is strong, the value of $K_{1/2}$ does not represent the true Michaelis-Menten constant.

The values of K_m and $V_{\max.}$ (the true maximum velocity) calculated for *p*-chlorophenylglucuronide enabled us to determine the dissociation constants

(K_I) of the complexes of β -glucuronidase with certain sugar acid inhibitors. The literature contains only one previous value of K_I , that for D-glucosaccharic acid by Karunairatnam & Levvy (1949). Their value, $6 \times 10^{-6} M$, was only about 1/15 of our value of $8.8 \times 10^{-4} M$. In their calculation of K_I from equation (3) when $V_{\max}/v = 2$, Karunairatnam & Levvy have actually used $K_{\frac{1}{2}v_{\text{opt}}}$ for K_m and V_{opt}/v for $V_{\max}/v$.

Three sugar acids and one sugar acid lactone were tested for inhibitory action on β -glucuronidase (see Table 6). In agreement with Karunairatnam & Levvy (1949), D-glucosaccharic acid was found to be the most powerful inhibitor, its affinity for the enzyme being 13 times that of D-glucuronic acid and 40 times that of mucic acid. Karunairatnam & Levvy do not clearly distinguish in their paper between glucurone and glucuronic acid, but they have informed us, in a personal communication, that their method of making up the glucurone solution very probably converted it to glucuronic acid; it appears very probable that the inhibition they record for glucurone was due to glucuronic acid. We found that D-glucurone did not inhibit β -glucuronidase. On theoretical grounds it might be expected

that the enzyme would be inhibited by glucuronic acid but not by glucurone, because these compounds have totally different structures, glucuronic acid (II) being a pyranose (Challinor, Haworth & Hirst, 1931; see also Smith, 1939), and glucurone (III) a furanose derivative (Smith, 1944).

The sodium D-glucuronate used by us was a mutarotated form containing the α - and β -isomers and we cannot say whether the inhibition is due to both forms or due to the β -form only (cf. I which is a β -glucuronide, a normal substrate for β -glucuronidase). Since D-glucosaccharic acid (IV) is a powerful inhibitor of the enzyme, it appears probable that a strong inhibitor should possess two carboxyl groups and chain of CHOH groups of similar configuration to that of the glucuronide (I). D-Glucurone has neither a carboxyl group nor the necessary hydroxyl groups on C atoms 3 and 4. Mucic acid (V) has two carboxyl groups, but not the necessary configuration of CHOH groups and is less inhibitory than glucuronic acid, which has one carboxyl plus the necessary configuration of CHOH groups.

SUMMARY

1. It has been shown that β -glucuronidase can be determined spectrophotometrically using *p*-chlorophenylglucuronide as substrate, since *p*-chlorophenol in alkali has absorption bands at 245 and 298 $m\mu$, where the absorption of the glucuronide is negligible.

2. The method has been applied to an ox spleen glucuronidase preparation and the results examined by the analytical methods of Lineweaver & Burk (1934). The inhibition of the enzyme by excess substrate has been shown to be competitive.

3. Various constants for the action of β -glucuronidase on *p*-chlorophenylglucuronide have been calculated.

4. Inhibition of the enzyme by D-glucosaccharic, mucic and D-glucuronic acids and D-glucurone has been studied. Glucurone was found to be non-inhibitory and the powerful inhibitory action of glucosaccharic acid has been confirmed. These results have been discussed.

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Studies in Detoxication

36. A NOTE ON THE GLUCURONIDES OF BENZENEAZOPHENOL AND BENZENEAZORESORCINOL

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Salant & Bengis (1916) found that a number of fat-soluble dyes administered to rabbits were excreted in the urine and bile, besides being deposited in adipose tissues. Since no fat was excreted in the urine with these dyes, these workers concluded that they were converted in the body into water-soluble substances, probably ethereal sulphates and glucuronides. Benzeneazophenol (oil yellow) and benzeneazoresorcinol (Sudan G 35) were fed to rabbits and the urine was examined for metabolites. Glucuronides were isolated in both cases and although melting points were recorded, the compounds were not analysed or properly described.

It appeared from the paper of Salant & Bengis that the glucuronides of benzeneazophenol and benzeneazoresorcinol would be compounds readily prepared by acidification of urine, and we thought that they might perhaps be suitable substrates for the assay of β -glucuronidase, if the glucuronides were less highly coloured than the free azophenols. We have therefore prepared and characterized these glucuronides and examined their possibilities as substrates for glucuronidase.

We have assumed that the monoglucuronide obtained from benzeneazoresorcinol is 4-benzeneazo-3-hydroxyphenylglucuronide (I) on analogy

with the biosynthetic monoglucuronides of 4-chlororesorcinol (Dodgson & Williams, 1949) and 2:4-dihydroxyacetophenone (Dodgson, 1950), which have been proved to be *para*-substituted phenyl glucuronides (see also Dodgson, Smith & Williams, 1950).

EXPERIMENTAL

4-Benzeneazophenyl- β -D-glucuronide. Benzeneazophenol (m.p. 151–152°; 2 g.) was fed to a rabbit (weight, 3 kg.). After 3 hr. the bright-yellow urine (50 ml.), which gave negative tests for aromatic amino groups and reducing substances but positive ones for glucuronic acid, was acidified with HCl. A small pale-yellow crystalline precipitate of the glucuronide separated; this, however, redissolved on further acidification.

To isolate the glucuronide, the urine was made faintly acid to litmus and extracted three times with a mixture of 1 vol. ethanol to 3 of ether. The extracts were evaporated to a syrup to which was added Na_2SO_4 to form a paste. The latter was then stirred repeatedly with small amounts of the ethanol-ether mixture until all the yellow material had been extracted. Evaporation of this solution gave