tyrosine fluorescence was detected in low yield with band maximum at 304 m μ only in the absence of tryptophan.

3. In proteins containing tryptophan the fluorescence spectrum of this residue alone is observed, irrespective of the wavelength of the exciting light, even in those cases where tyrosine predominates in absorption. The band maxima vary in wavelength between 328 and 342 m μ , and the overall quantum yields 3.8–15.2 % in water.

4. Detailed examination of the change in tryptophan quantum yield in the excitation region between 280 and 310 m μ reveals that energy transfer from tyrosine to tryptophan does not take place to any appreciable extent. The similarity of all protein-excitation spectra with that of either tyrosine or tryptophan shows that light absorbed by cystine or the peptide bond does not contribute to protein fluorescence.

5. Modification of the protein structure by 8-m-urea produces large and in some cases reversible changes in tryptophan fluorescence, leading to similar yields and fluorescence spectra in most of the proteins examined. The slight structural change involved in the activation of chymotrypsinogen increases the quantum yield by 32 %. Propane-1:2-diol increased the yield of all the proteins except the serum albumins and lysozyme.

6. The absence of tyrosine fluorescence in many proteins is explained by the quenching action of the tyrosyl-carboxylate hydrogen bond, and is correlated with the pK values for the phenolic ionization. The role of this linkage in the main-

tenance of the protein tertiary structure is briefly discussed.

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The Biochemistry of Aromatic Amines

7. THE ENZYMIC HYDROLYSIS OF AMINONAPHTHYL GLUCOSIDURONIC ACIDS*

By E. BOYLAND AND K. WILLIAMS

Chester Beatty Institute, Institute of Cancer Research: Royal Cancer Hospital, London, S.W. 3

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As 2-naphthylamine induces cancer of the bladder only, in men exposed to the amine, the actual carcinogen is probably a metabolite of the amine (Boyland, 1958). When tested by implantation in the mouse bladder 2-naphthylamine was much less carcinogenic than one of its metabolites, 2-amino-1-naphthol (Bonser, Clayson, Jull & Pyrah, 1952; Bonser, Clayson & Jull, 1958). However, the 2-amino-1-naphthol has been detected

in fresh urine only in conjugated forms (Boyland, 1958) and Boyland (1956) has suggested that it is necessary for the conjugates to be hydrolysed before carcinogenesis occurs. 2-Amino-1-naphthyl sulphate is not hydrolysed by urinary sulphatase (Boyland, Manson, Sims & Williams, 1956) and is not carcinogenic in the mouse bladder (Bonser, Bradshaw, Clayson & Jull, 1956). The corresponding glucosiduronic acid, however, can be hydrolysed by mammalian β -glucuronidase preparations (Boyland & Manson, 1957) and is carcinogenic in the

* Part 6: Boyland & Williams (1959).

mouse bladder (Allen, Boyland, Dukes, Horning & Watson, 1957). Other ways by which 2-amino-1-naphthol might be released in the bladder include the hydrolysis of bis-(2-amino-1-naphthyl) phosphate, which Troll, Belman & Nelson (1959) have detected as a metabolite of 2-naphthylamine in dog urine, and the removal of the sulphate group from 2-amino-1-naphthyl sulphate by means other than by aryl sulphatase.

Since β -glucuronidase may play a part in the induction of bladder cancer after exposure of subjects to 2-naphthylamine, the properties of human urinary β -glucuronidase and its action upon some metabolites of 2-naphthylamine have been investigated.

MATERIALS AND METHODS

Glucuronidase substrates. Phenolphthalein mono- β -glucosiduronic acid (Sigma Chemical Co.) and 8-hydroxyquinoline glucosiduronic acid (California Foundation for Biochemical Research) were commercial samples. 2-Acetamido-1-naphthyl glucosiduronic acid, 2-acetamido-6-naphthyl glucosiduronic acid and 2-amino-1-naphthyl glucosiduronic acid were prepared according to Boyland & Manson (1957). 1-Naphthyl glucosiduronic acid was biosynthesized as described by Berenbom & Young (1951).

Other substrates. 2-Amino-1-naphthyl sulphate was prepared by the persulphate oxidation method of Boyland, Manson & Sims (1953), and 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate) according to Roy (1953). 2-Amino-1-naphthyl phosphate was prepared by a perphosphoric acid oxidation of 2-naphthylamine (Boyland & Manson, 1957).

Inhibitors. Ammonium glucosaccharo-1 $\rightarrow$ 4-lactone, the 6-methyl and 6-ethyl esters and free glucosaccharo-3 $\rightarrow$ 6-lactone were commercial samples from Kemball, Bishop and Co., London.

Determination of enzyme activity

Glucuronidase. The activity of this enzyme is expressed in units: one unit is that which liberates 1 μ g. of phenolphthalein in 1 hr. at 37° from phenolphthalein glucosiduronic acid (0.33 mM, pH 4.5), the liberated phenolphthalein being determined colorimetrically at pH 10.5 (Boyland, Wallace & Williams, 1957). 8-Hydroxyquinoline, 1-naphthol, 2-acetamido-1-naphthol and 2-acetamido-6-naphthol were estimated with the Folin-Ciocalteu reagent (Bray & Thorpe, 1954), but 2-amino-1-naphthol could not be determined by this method because it is so easily oxidized. The hydrolysis of 2-amino-1-naphthyl glucosiduronic acid was measured with the Aminco-Bowman spectrophotofluorometer (see below).

Sulphatase. Nitrocatechol liberated from the corresponding sulphate was estimated by the spectrophotometric method described by Dodgson, Melville, Spencer & Williams (1954). Attempts were made to determine the hydrolysis of 2-amino-1-naphthyl sulphate by the benzidine method of Dodgson & Spencer (1953) and by the spectrophotofluorimeter.

Estimations with the Aminco-Bowman spectrophotofluorimeter

The solution to be examined is placed in a quartz cell and a monochromator is used to select a suitable activating wavelength from a xenon arc lamp. Fluorescent light emitted by the sample at right angles to the activating beam is passed through another monochromator on to a photoelectric cell which then excites a galvanometer through a photomultiplier. The instrument permits the detection and estimation of small amounts of fluorescent materials but certain difficulties arise when it is used for the estimation of enzymic activity as in the present case. The most serious is the effect of protein upon the fluorescence. The slight cloudiness of the enzyme solution causes serious errors due to 'scatter' peaks appearing in the fluorescent spectrum. Further, the absorption of ultraviolet light by protein results in a decrease of the apparent fluorescence of the material being examined. These difficulties were overcome by precipitating the protein in the incubation by the addition of 4 vol. of ethanol. The supernatant gave very little scatter and the absorption effect of protein was almost eliminated. The instrument employs a single beam and standard solutions were used to set the reading of the spectrophotofluorimeter during each experiment. Since the unused substrate is being estimated standards are provided by the controls, where ethanol is added immediately after the mixing of enzyme and substrate.

All the 2-amino-1-naphthyl derivatives examined had similar activation and fluorescent spectra, with maximal activation at 325–330 $m\mu$ and a fluorescent peak at 410 $m\mu$. The fluorescence of free 2-amino-1-naphthol itself can be seen if precautions are taken to prevent its oxidation. Addition of 4 vol. of ethanol to a solution of mM-2-amino-1-naphthol results in immediate destruction of the free phenol, but the conjugates are stable for at least 1 hr. For the enzyme experiments 0.25 ml. of enzyme solution in buffer was incubated with 0.25 ml. of a substrate solution, and the reaction stopped at the appropriate time by the addition of 2 ml. of ethanol. Appropriate controls were set up in which the enzyme and substrate were mixed just before the addition of the ethanol. The spectrophotofluorimeter was set at approximately 325 $m\mu$ activation wavelength and 410 $m\mu$ on the fluorescence monochromator and, with one of the controls as the standard fluorescent solution, the wavelength disks were adjusted to give the maximum reading on the photomultiplier galvanometer. The sensitivity of the instrument was then adjusted to give a fixed reading. These adjustments are necessary to correct for fluctuations in the instrument.

In order to check that the decrease in fluorescence was related to the hydrolysis of 2-amino-1-naphthyl derivatives, the hydrolysis of 2-amino-1-naphthyl phosphate by acid phosphatase was followed by this method and by estimation of the phosphate liberated (Fiske & Subbarow, 1925). The two methods were found to be in good agreement.

Anaerobic experiments

These were carried out in Thunberg tubes in an atmosphere of nitrogen. After incubation, 0.5 ml. of the medium was pipetted into 2 ml. of ethanol and the fluorescence of the supernatant determined.

Determination of kinetic constants

These were determined where applicable by the Lineweaver & Burk (1934) method. In the experiments in which the spectrophotofluorimeter was used the decrease in concentration of the substrate was measured and a considerable proportion had to be destroyed before accurate estimations of the hydrolysis could be obtained. In this case the Lineweaver-Burk plot could not be used because it makes the assumption that the concentration of the substrate is substantially constant throughout the experiment. The integrated Michaelis equation of Dixon & Webb (1958) was therefore used.

Inhibition constant of glucosaccharo-1 $\rightarrow$ 4-lactone. The K_i values for the lactone were determined by the method of Dixon (1953). At first, when low substrate concentrations were used, non-linear plots were obtained. It was later discovered that this was due to the extremely high affinity of this inhibitor for the enzyme, but this difficulty could be overcome by the use of high substrate concentrations for reasons which will be given later.

Concentration of the urinary enzymes

The β -glucuronidase activity of normal human urine is low (approx. 0.5–2 units/ml.) and the urine frequently contains endogenous inhibitors. Concentration of the enzyme by precipitation of the urine at pH 5.6 with 30% (v/v) of acetone was unsatisfactory as the proportion of the total activity precipitated varied from sample to sample.

The ultrafiltration method of Grant & Everall (1957) using 20 ft. of $\frac{3}{8}$ in. flat Visking tubing was more consistent. Cooled, fresh, human male urine (2 l., pooled samples) could be concentrated to 20–30 ml. overnight in the cold. The concentrates were dialysed overnight against distilled water in the cold and, after centrifuging to remove the deposit, preparations containing apparently more than 100% of the original activity of the urine were consistently obtained. Such concentrates had linear dilution-activity relationships showing that they were free from dissociable inhibitors or activators, and were used, unless otherwise stated, in the enzyme experiments described.

Preparation of a concentrate of β -glucuronidase from human kidney

The kidney (205 g., total activity 420 000 units) from a patient with hydronephrosis, which had been removed at operation by Mr D. M. Wallace, was suspended in 300 ml. of ice-cold acetone in a Waring-type blender. The suspended matter was filtered off and washed with acetone on the filter. The acetone-insoluble material gave 26 g. (total activity 406 000 units) of a buff powder on drying. A 10% (w/v) suspension was made of this powder in water, kept for 15 min. and centrifuged at 600 g for 15 min. The supernatant (12 vol.) was diluted with 3 vol. of ice-cold acetone and the precipitate removed by centrifuging after 15 min. A further 5 vol. of acetone was added to bring the acetone concentration to 40% (v/v). This second precipitate was spun down after 15 min. and washed with further acetone. The dried powder was used as a source of β -glucuronidase for comparison with that obtained from urine. The activity was 157 000 units/g., the total activity being approx. 55% of that in the acetone-dried kidney.

Paper electrophoresis

The urine concentrate was run for 18 hr. on a vertically held Whatman no. 100 paper (36 cm. $\times$ 5 cm.) at 110 v in the presence of 0.05 M-barbiturate buffer, pH 8.6, 0.1 M-sodium acetate, pH 4.5, or 0.1 M-sodium phosphate, pH 7. The papers were dried at room temperature and the protein was located by staining with bromophenol blue (see Flynn & de Mayo, 1951). Cellulose acetate electrophoresis strips (Oxoid) were also used at pH 8.6. Enzyme activity was determined by cutting the paper into 1 cm. strips and then incubating these strips with 0.33 mM-phenolphthalein glucosiduronic acid in 0.1 M-acetate buffer, pH 4.5, for 18 hr. at 37°.

Cellulose-column chromatography

Carboxymethylcellulose suitable for columns was prepared according to Peterson & Sober (1956). A 10 cm. $\times$ 1.75 cm. diameter column, made up in 0.1 M-sodium phosphate buffer, pH 6, was used. The column was loaded with the urine concentrate and eluted with 0.02 M-phosphate buffer, pH 7.5. After overnight dialysis against distilled water, the fractions were incubated with 0.33 mM-phenolphthalein glucosiduronic acid in 0.1 M-acetate buffer, pH 4.5, for 18 hr. at 37° to determine the enzyme-elution band. The protein content of the fractions was estimated by the ultraviolet-absorption method of Warburg & Christian (1941).

RESULTS

Properties of the urinary β -glucuronidase

When the β -glucuronidase activity of whole urine and the enzyme concentrate were measured over 18 hr. at 37.5°, with phenolphthalein mono- β -glucosiduronic acid (4 mM) as substrate, the optimum pH in 0.1 M-acetate buffer was 4.5 for whole urine and 5.0 for the enzyme concentrate. The optimum pH for hydrolysis of 2-amino-1-naphthyl glucosiduronic acid (0.5 mM) by the concentrate under the same conditions was 4.6 (Figs. 1, 2).

Maximum activity of the concentrate occurred at a substrate concentration of 4 mM-phenolphthalein glucosiduronic acid in 0.1 M-acetate at pH 4.5 (Fig. 3). At this optimum substrate concentration the effects of enzyme concentration and time of incubation on the amount of phenolphthalein liberated were determined at pH 4.5. The relationship was linear up to concentrations of approx. 100 units/ml. (1 hr. incubation) and for incubations of up to 20 hr. duration with enzyme activity between 1 and 4 units/ml. Fig. 3 also shows that the hydrolysis of 2-amino-1-naphthyl glucosiduronic acid is directly proportional to the substrate concentration. The reaction is first-order with respect to the substrate concentration, showing that $s \ll K_s$ (equilibrium constant). The substrate concentration (s) is limited by the low solubility of the 2-amino-1-naphthyl glucosiduronic acid.

Talalay, Fishman & Huggins, (1946) found a pH optimum of 4.5 for the tissues of the mouse and rat with phenolphthalein glucosiduronic acid as substrate, but Mills & Paul (1949) reported the presence of two peaks at pH 4.4 and 5.2 with ox-spleen β -glucuronidase.

Enzyme multiplicity. No indication of the presence of more than one β -glucuronidase was obtained from the pH curve (Fig. 1) or by use of paper electrophoresis or carboxymethylcellulose columns (Fig. 4). Further, the enzyme obtained from human kidney was very similar to the urinary

β -glucuronidase in its behaviour towards several substrates (Table 1). This would be unlikely if there were more than one enzyme present in either source.

Activation energy. β -Glucuronidase activity at pH 4.5 in 0.1 M-acetate with 4 mM-phenolphthalein glucosiduronic acid as substrate was measured over the range 25–41°. By using the Arrhenius (1889) equation (Fig. 5) the activation energy was found to be 13 600 cal./mole.

Fig. 1. pH-Activity curves for the β -glucuronidase of a human urine concentrate in the presence of 0.1 M-acetate buffer. ●, Phenolphthalein mono- β -glucosiduronic acid (4 mM); ○, 2-amino-1-naphthyl glucosiduronic acid (0.5 mM). Incubation was for 18 hr. at 37.5°. An enzyme concentration that liberated approximately 1.5 μ g. of aglycone/ml./hr. at pH 5 was used with phenolphthalein glucosiduronic acid and a concentration that liberated 20–25 μ g./ml./hr. at pH 4.6 with 2-amino-1-naphthyl glucosiduronic acid.

Fig. 2. pH-Activity curve for the β -glucosiduronidase of whole urine in the presence of 0.1 M-acetate buffer. The substrate was 4 mM-phenolphthalein mono- β -glucosiduronic acid and incubation was for 18 hr. at 37.5°. Activity of the urine was 1–1.5 units/ml.

Fig. 3. Substrate concentration-activity curve for the β -glucuronidase of human urine concentrate in the presence of 0.1 M-acetate buffer, pH 4.5. Substrates: ○, phenolphthalein mono- β -glucosiduronic acid; △, 2-amino-1-naphthyl glucosiduronic acid; incubation was for 18 hr. at 37.5°. The enzyme concentrations used were similar to those in Fig. 1. (For comparison of the relative rates of hydrolysis of the two substrates see text.)

Fig. 4. Elution of β -glucuronidase activity of urine concentrate from a carboxymethylcellulose column with 0.02 M-phosphate buffer, pH 7.5. Each fraction was 3.9 ml. Fraction 2 contained 52 units of enzyme and 9.2 mg. of protein. A, β -Glucuronidase activity; B, protein concentration.

Kinetic constants. Michaelis constants and $V_{\max}$ for a number of glucosiduronic acids were determined at pH 4.5 in 0.1 M-acetate buffer after incubation for 18 hr. at 37.5°. The results are shown in Table 1.

No hypothesis on the nature of the enzyme action can be advanced since the quantitative effect of the aromatic radicals upon the ether link of the glucosiduronic acid is difficult to estimate with the substrates used. It is, however, of interest that with the exception of 1-naphthyl glucosiduronic acid the K_m values are more influenced by the nature of the substrate than are the $V_{\max}$ values. The aglycone has more effect on the free substrate than on the enzyme-substrate complex.

The rate of enzymic hydrolysis of 2-amino-1-naphthyl glucosiduronic acid was measured by determining the destruction of the substrate, and the integrated Michaelis equation of Dixon & Webb (1958) was used in an attempt to determine K_m and $V_{\max}$ for this substrate. Here the assumption was made that the products of the reaction do not have a significant effect upon the reaction rate, but this

might not be justifiable, since a considerable proportion (30–50 %) of the substrate was destroyed. However, one of the products of the reaction, 2-amino-1-naphthol, is easily oxidized so that it is probably destroyed during an aerobic incubation for 18 hr. Experiments were also carried out under anaerobic conditions. Under both conditions slight positive slopes were obtained (Fig. 6), whereas a negative slope would be expected for the normal case. Dixon & Webb (1958) have, however, shown that in regions where the substrate concentration is not much greater than the Michaelis constant a line with zero slope is obtained. The concentration of 2-amino-1-naphthol glucosiduronic acid is restricted to low values by its limited solubility. The experiments appear therefore to have been carried out in the region where zero slopes might be obtained, and the values of K_m or $V_{\max}$ for 2-amino-1-naphthyl glucosiduronic acid cannot be determined by this method alone. The slight increase in the reaction rate under aerobic conditions, as compared to the anaerobic experiments, may be a result of the removal of 2-amino-1-

Fig. 5. Effect of temperature on the activity of β -glucuronidase on 4 mM-phenolphthalein mono- β -glucosiduronic acid. Time of incubation was 18 hr. in 0.1 M-acetate, pH 4.5. The results are plotted in the form $1/T$ (°K) against $\log v$ for calculation of the activation energy of the enzyme.

Fig. 6. Effect of the concentration of 2-amino-1-naphthyl glucosiduronic acid upon the activity of the β -glucuronidase of human-urine concentrate in the presence of 0.1 M-acetate, pH 4.5. The results are plotted in the form:

$$\frac{2.303}{t} \log \frac{s_0}{s_0 - y}$$

against y/t , where t is the time, s_0 the original substrate concentration and $s_0 - y$ the substrate remaining at the end of the experiment, in order to make use of an integrated Michaelis equation (see text). Incubation was for 18 hr. at 37.5°; ●, aerobic conditions; ○, anaerobic conditions.

Table 1. *Hydrolysis of glucosiduronic acids by the β -glucuronidase from human male urine and human kidney*

The $V_{\max}$ values are quoted relative to that obtained with the urine enzyme and phenolphthalein glucosiduronic acid. Incubates contained 0.1 M-acetate buffer, pH 4.5; the time of incubation was 18 hr. at 37.5°. Because of the limited solubility of the substrate and its low affinity for the glucuronidase, the K_m and $V_{\max}$ values could not be determined for 2-amino-1-naphthyl glucosiduronic acid.

Substrate	$10^4 K_m$ (M)		$V_{\max}$	
	Urine	Kidney	Urine	Kidney
Phenolphthalein glucosiduronic acid	3.3	3.6	100	105
8-Hydroxyquinoline glucosiduronic acid	8.3	7.7	114	110
2-Acetamido-6-naphthyl glucosiduronic acid	13.8	15.4	85	91
2-Acetamido-1-naphthyl glucosiduronic acid	50	50	99	102
1-Naphthyl glucosiduronic acid	29.4	—	32	—

naphthol. Some quenching of the fluorescence of the substrate by the products of the reaction could result in the slight positive slopes. It was not possible to determine K_i (which might approximate to K_m and thus with the $V_{\max}/K_m$ value already obtained give $V_{\max}$.) for 2-amino-1-naphthyl glucosiduronic acid when acting as a competitive inhibitor for phenolphthalein glucosiduronic acid. The low solubility and affinity of the 2-amino-1-naphthyl glucosiduronic acid did not allow sufficient inhibition to be obtained for estimation of this kinetic constant. This result supports the reasons given for the almost zero slopes obtained with the integrated Michaelis equation.

Despite the low affinity of the β -glucuronidase for 2-amino-1-naphthyl glucosiduronic acid, this substrate has an appreciable rate of hydrolysis. In 0.1M-acetate, pH 4.5, the rate of substrate destruction, on a molar basis, of phenolphthalein glucosiduronic acid (0.33 mM) and 2-amino-1-naphthyl glucosiduronic acid (0.33 mM) was 1:1.26 for 18 hr. incubations, under aerobic conditions. Under anaerobic conditions the ratio was 1:0.93.

Inhibition of the β -glucuronidase by glucosaccharo-1 $\rightarrow$ 4-lactone and the 6-methyl and 6-ethyl esters of the lactone

Glucosaccharo-1 $\rightarrow$ 4-lactone is a powerful competitive inhibitor of mouse-liver β -glucuronidase (Levvy, 1952) and its action upon the human urinary enzyme was examined. Initial attempts to determine the inhibition constant by the method of Dixon (1953) gave non-linear plots. If phenolphthalein was incubated with the urine concentrate as pH 4.5 complete recoveries as shown colorimetrically were obtained. Combination of substrate and inhibitor (see Friedenwald & Maengwyn-Davies, 1954) did not seem a probable explanation because the concentration of substrate was over 100 times as great as that of the inhibitor and each inhibitor molecule would have to combine with several molecules of substrate to give the degree of inhibition obtained. Thus inhibition was almost certainly due to enzyme-inhibitor interaction.

The Michaelis-Menten equation, upon which the normal methods of determining equilibrium constants are based, makes the assumption that the concentration of the substrate or inhibitor combining with the enzyme is very much greater than the concentration of the enzyme-active sites. Glucosaccharo-1 $\rightarrow$ 4-lactone has a very high affinity for β -glucuronidase and thus could be acting in a pseudo-irreversible manner under the conditions (substrate concentration 0.25, 0.5 and 1 mM; inhibitor concentrations 0.2–2 μ M) used in the initial attempts to determine K_i .

Extensions of the original Michaelis-Menten treatment to include such cases have been put

forward by Strauss & Goldstein (1943), Goldstein (1944) and Ackermann & Potter (1949). The last-named authors describe a simple graphical method for determining whether the Michaelis-Menten equation is applicable to a particular case. Plotting of the rate of reaction against the enzyme concentration (substrate 75 μ M, inhibitor 1 μ M) in the presence and absence of inhibitor gave the pseudo-irreversible inhibitor plot of Ackermann and Potter, i.e. the inhibitor line cuts the X axis to the right of the origin.

For reasons of economy low concentrations of phenolphthalein glucosiduronic acid well below the optimum had been employed. Substrate concentrations nearer the optimum, however, enable smaller enzyme concentrations to liberate sufficient phenolphthalein for accurate estimation. Higher concentrations of substrate also allow the use of higher inhibitor concentrations. Thus the relative enzyme concentration is decreased and the inhibitor concentration increased so that the system moves away from the pseudo-irreversible zone. An Ackermann-Potter graph at high substrate concentrations (substrate 3.75 mM, inhibitor 10 μ M) now gave the plot for reversible inhibition, i.e. both in the absence and presence of the inhibitor, straight lines which pass through the origin were obtained.

When substrate concentrations of 3.75 and 1.88 mM and inhibitor concentrations (i) of 10–50 μ M were used linear plots of $1/v$ against i were obtained, showing glucosaccharo-1 $\rightarrow$ 4-lactone to be a competitive inhibitor (Dixon, 1953) with a K_i of 1.6 μ M for human urinary β -glucuronidase.

From these results it appears that the original system used for determining K_i was in the region where the assumption made in the Michaelis-Menten equation was becoming unjustifiable; for by altering the concentrations of enzyme, substrate and inhibitor (which for practical reasons are dependent upon each other) it was possible to push the systems into regions where (a) the Michaelis-Menten equation was applicable, (b) where the enzyme-inhibitor reaction was pseudo-irreversible.

Glucosaccharo-1 $\rightarrow$ 4-lactone has been administered to patients from whom bladder tumours have been removed in order to reduce the urinary β -glucuronidase activity (Boyland *et al.* 1957). The effect of the lactone was measured by determining the fall in β -glucuronidase activity of the urine at the optimum pH 4.5. Now the pH of human urine (Dr H. Davies, personal communication) can vary from 5 to above 7, the mode being about 5.8. Figs. 7 and 8 show the effect of pH upon the inhibition by saccharolactone of the β -glucuronidase in a urine concentrate. The inhibition falls away from that at the optimum pH. However, it is still appreciable at pH 5.8 and the saccharolactone exerts its greatest action where it is most required,

i.e. when the enzyme is most active (Boyland, Davies & Williams, 1959). The measurement of the activity of the urine at pH 4.5 can, however, give a misleading impression of the actual activity in the bladder.

The esters of glucosaccharo-1 $\rightarrow$ 4-lactone were much less potent inhibitors of β -glucuronidase. The 6-methyl ester was shown by the method of Dixon (1953) to be non-competitive with K_i 1 mM. The 6-ethyl ester was not an inhibitor.

In all the inhibitor experiments the lactones were made up just before use. Incubation was for

1 hr. in 0.2M-acetate buffer, the reaction being stopped and the phenolphthalein colour developed by the addition of 10% (anhydrous w/v) sodium carbonate solution.

Sulphatase

Boyland *et al.* (1956) showed that no free 2-amino-1-naphthol could be detected by the Folin-Ciocalteu reagent after incubation of 2-amino-1-naphthyl sulphate with urinary sulphatase and this observation has been confirmed by independent methods. Attempts were made to estimate sulphate liberation by the benzidine method of Dodgson & Spencer (1953), but the sulphate liberated from 0.5 mM-2-amino-1-naphthyl sulphate at pH 5.6 in 0.1M-acetate buffer was less than 1% of that liberated from nitrocatechol sulphate under the optimum conditions (0.03M, pH 5.6, 0.25M-buffer; Dodgson & Spencer, 1957). The spectrophotofluorimetric estimations also showed that the rate of destruction of 2-amino-1-naphthyl sulphate was less than 1% of nitrocatechol sulphate under these conditions.

DISCUSSION

After consideration of the carcinogenic properties of metabolic derivative of aromatic amines, Boyland (1959) suggested that 2-amino-1-naphthyl glucosiduronic acid or bis-(2-amino-1-naphthyl)phosphoric ester could be the causes of bladder cancer resulting from exposure to 2-naphthylamine. The actual carcinogen was probably 2-amino-1-naphthol (or an oxidation product) liberated in the bladder by the action of the urinary β -glucuronidase, or other hydrolytic process. Use of a concentrate of human urinary β -glucuronidase has shown that the enzyme will hydrolyse 2-amino-1-naphthyl glucosiduronic acid at about the same rate as the phenolphthalein conjugate. Further evidence was obtained that the 2-amino-1-naphthyl sulphate was inert to the action of urinary sulphatases, this being a possible explanation for its not having a carcinogenic action in the mouse bladder as does the glucosiduronic acid (Allen *et al.* 1957).

The inhibition of human β -glucuronidase at low concentrations of substrate and of glucosaccharo-1 $\rightarrow$ 4-lactone has been found to be pseudo-irreversible. However, unlike the case of physostigmine and cholinesterase considered by Strauss & Goldstein (1943), a pseudo-irreversible reaction would not occur in the urine of patients with bladder cancer undergoing prophylactic treatment with saccharolactone. β -Glucuronidase is present in low concentration in urine whereas cholinesterase is very highly concentrated at the motor end-plates. Despite its very high affinity therefore it would seem that an inhibitor of increased affinity would be still more effective in suppressing the β -gluc-

Fig. 7. Effect of pH upon the inhibition of the β -glucuronidase of human-urine concentrate by glucosaccharo-1 $\rightarrow$ 4-lactone. Incubation was for 1 hr. at 37.5°, and the buffer was 0.2M-acetate. Substrate 330 μ M-phenolphthalein glucosiduronic acid, inhibitor concentration 10 μ M. O, pH-activity curve in the absence of inhibitor; Δ , pH-activity curve in the presence of inhibitor; these results are expressed as a percentage of the maximum uninhibited activity; $\blacktriangle$, inhibition expressed as a percentage of the activity at the same pH in the absence of inhibitor.

Fig. 8. As Fig. 7 but with 330 μ M-2-amino-1-naphthol glucosiduronic acid used as the substrate. In calculating the activity, account has been taken of the destruction of a large proportion of the total substrate concentration (see text). Incubation was for 18 hr.

uronidase activity of urine. The main disadvantage of saccharolactone is, however, that it is so rapidly metabolized by the body that only a small proportion of the dose is excreted.

Consideration of the effect of pH upon the total activity and the percentage inhibition must be given since both fall sharply away from values at the optimum pH. In the testing of urine for activity its original pH as well as the activity at pH 4.5 should be noted in order that a more exact indication of the activity *in vivo* is obtained. Measurement at the pH of the urine would be difficult because of its low activity.

There has been no evidence of the abnormality in the mammalian enzyme comparable with that reported for the β -glucuronidase of *Cellana tramoseria* (Cox, 1959). The pH optimum of the concentrate remains constant with variation in substrate concentration. There was, however, a slight shift in the optimum pH from 4.5 to 5 on concentration of the enzyme. No evidence for the presence of more than one enzyme was discovered.

Structure of ammonium glucosaccharo-1 $\rightarrow$ 4-lactone

Although the lactone is such a potent inhibitor of β -glucuronidase it has been assigned a 1:4 ring (Smith, 1944), whereas bornyl glucosiduronic acid (Pryde & Williams, 1933) and 2-naphthyl glucosiduronic acid (Tsou & Seligman, 1952) have been shown, by independent methods, to possess 1:5-pyranoside rings. Marsh & Levvy (1958) have also synthesized aryl β -D-glucopyranosiduronic acids by the same general method (catalytic oxidation of hexosides) as Tsou & Seligman (1952) and show that the hydrolysis of these conjugates by β -glucuronidase is inhibited by saccharolactone. The evidence presented by Levvy (1952) and that in this paper show that only slight changes in the saccharolactone molecule greatly reduce its affinity for the enzyme. This stereochemical specificity may throw some doubt upon the ring structure given by Smith (1944), since this has not been confirmed by independent methods, unlike the structure of the aryl β -D-glucopyranosiduronic acids.

SUMMARY

1. β -Glucuronidase of human urine, prepared by ultrafiltration and dialysis, hydrolyses the *O*-glucosiduronic acid derivatives of phenolphthalein, 8-hydroxyquinoline, 2-acetamido-6-naphthol, 2-acetamido-1-naphthol, 1-naphthol and 2-amino-1-naphthol, although 2-amino-1-naphthyl glucosiduronic acid has a lower affinity than the other substrates.

2. The theoretical maximum velocity of hydrolysis ($V_{\max}$) for 2-amino-1-naphthyl glucosiduronic acid could not be determined; that for 1-naphthyl

glucosiduronic acid was a third of that of other substrates.

3. The affinity of glucosaccharo-1 $\rightarrow$ 4-lactone for β -glucuronidase is 1000 times as great as that of its 6-methyl and 6-ethyl esters. The inhibition of β -glucuronidase by glucosaccharo-1 $\rightarrow$ 4-lactone is much greater in the region of the optimum pH of the enzyme.

4. The resistance of 2-amino-1-naphthylsulphate to hydrolysis by aryl sulphatase has been confirmed.

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Cobalamin and the Synthesis of Methionine by Ultrasonic Extracts of *Escherichia coli*

By J. R. GUEST, C. W. HELLEINER,* M. J. CROSS† AND D. D. WOODS
Microbiology Unit, Department of Biochemistry, University of Oxford

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The synthesis of methionine from homocysteine and serine by *Escherichia coli* has been studied in these laboratories with suspensions of intact organisms (Gibson & Woods, 1960) and with enzymic extracts (Cross & Woods, 1954; Szulmajster & Woods, 1960; Kisliuk & Woods, 1960). With whole organisms strong presumptive evidence was obtained that some form of folic acid was required as coenzyme for the C₁-unit transfer and this was confirmed by the studies with enzyme preparations, though tetrahydropteroylglutamic acid and other forms of folic acid tried were not effective sources of the folic acid coenzyme (Cross & Woods, 1954; Szulmajster & Woods, 1955, 1960). Extracts of acetone-dried organisms were, however, activated by tetrahydropteroylglutamic acid provided that the organisms had been harvested from a medium containing cobalamin (Kisliuk & Woods, 1957, 1960).

Using an auxotrophic strain of *E. coli* requiring methionine or cobalamin for growth, Gibson & Woods (1952, 1960) found that washed suspensions of organisms harvested from a medium devoid of cobalamin did not synthesize methionine unless the vitamin was added. The formation of methionine by suspensions of a number of strains of *E. coli* not requiring cobalamin for growth was also increased markedly by the vitamin, the largest effect (over fivefold) being obtained with an auxotrophic strain (PA15) with a growth requirement for serine or

glycine. Most of the work with enzymic extracts quoted above was done with extracts of acetone-dried powders of this strain and no direct effect of cobalamin on the activity of the extracts was found.

In the present work the primary enzyme preparations were made by ultrasonic vibration instead of by drying with acetone. An effect of cobalamin was at once apparent under all conditions with a mutant requiring cobalamin or methionine for growth (and grown with methionine). Cobalamin now also activated methionine synthesis under certain conditions with strain PA15; it enabled tetrahydropteroylglutamate to serve as a source of the folic acid coenzyme also required in the reaction. Brief accounts of part of this work have already been published (Helleiner & Woods, 1956; Helleiner, Kisliuk & Woods, 1957; Woods, 1958). Towards the end of this work a brief account appeared of a cobalamin-containing enzyme, forming part of the complex of enzymes (in a cobalamin auxotroph of *E. coli*), which catalyses the transfer of C-3 of serine to homocysteine (Hatch, Takeyama & Buchanan, 1959).

There have been several investigations of the formation of methionine from homocysteine by enzymic extracts of chicken, sheep and pig liver with serine or formaldehyde as donor of the C₁ unit (Doctor, Patton & Awapara, 1957; Nakao & Greenberg, 1958; Stevens & Sakami, 1959). No effect of cobalamin was described; tetrahydropteroylglutamic acid was an effective source of folic acid with sheep- and pig-liver preparations and pteroylglutamic acid itself was active with the enzyme from chicken liver.

* Post-doctoral Fellow, National Research Council of Canada at the time of this work.

† Guinness Research Fellow in Microbiological Biochemistry at the time of this work.