obtained and parallel determinations of inorganic chloride must be carried out. Using the micromethod, a direct determination of $1\cdot0\,\mu$g. chlorine is possible. The ultramicro method is intended for use with amounts of tissue providing less than $1\cdot0\,\mu$g. of chlorine, e.g. tissue slices. It is hoped that, by reducing the size of the apparatus and by modifying the determination of iodine, amounts of chlorine down to $0\cdot1\,\mu$g. may be determined. Such a technique might be useful for investigations on the metabolism of DDT in insects.

It should be pointed out here that the application of the method to substances other than those specifically mentioned may be limited by their volatility. For example, consistently low results were obtained when estimations were attempted on chloroacetamide. This was attributable to volatilization of the substance before complete oxidation could take place.

SUMMARY

1. Methods are described for the micro and ultramicro determination of chlorine in DDT and related compounds, applicable to amounts of 1–120 μg. and 0·25–1·0 μg. of chlorine, respectively.

2. The techniques have been successfully applied to the analysis of tissues containing these substances.

My thanks are due to Prof. G. R. Cameron, F.R.S. for his kindness and interest in this work and the experiments still in progress; to Prof. C. Rimington for his advice and criticism; to Dr D. R. Wilkie, University College, London, Messrs J. S. Pizey and J. C. Godding, and finally to Mr J. Trendall, glassblower, University College, London, without whose skill and ingenuity this work would have been made immeasurably more difficult.

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Studies in Detoxication

25. THE CHARACTERIZATION OF PHENYLGLUCURONIDE, AND ITS RATE OF HYDROLYSIS COMPARED WITH THAT OF PHENYLSULPHURIC ACID

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The present work was carried out for two reasons. First, investigations in this laboratory on the metabolism of benzene showed that 11% of orally administered benzene was excreted by rabbits as glucuronides (Porteous & Williams, 1949*a*), and Schmiedeberg (1881) isolated from the urine of a benzene-fed dog a crystalline substance which was probably phenylglucuronide. We therefore required phenylglucuronide as a reference compound for further investigations on benzene. Secondly, phenylglucuronide is used as a standard substrate for the assay of β-glucuronidase in tissues (see Kerr, Graham & Levvy, 1948; Mills, 1948). After a search of the literature we felt that phenylglucuronide had not been properly described.

Phenyl-β-D-glucuronide was first isolated by Külz (1890) from the urine of rabbits receiving phenol by injection. His material melted at 148°, but he attributed the wrong formula, $C_{12}H_{16}O_7$, in accordance with his analytical figures. It was synthesized from phenol and acetobromoglucurone by Neuberg & Niemann (1905), who described it as anhydrous, m.p. 150–151°, $[\alpha]_D^{17°}$ $-83\cdot3°$ (solvent not mentioned). For material isolated from the urine of a sheep fed with phenol, Salkowski & Neuberg (1906) gave m.p. 148–150°, $[\alpha]_D$ $-81\cdot9°$ (solvent not mentioned) and analytical figures for the

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anhydrous glucuronide ($C_{12}H_{14}O_7$). Masamune (1933), who isolated the compound from rabbit urine after phenol feeding, dried it *in vacuo* at 78° and his analytical figures agreed with those for anhydrous phenylglucuronide. He gave m.p. 160° (decomp.), but quoted no specific optical rotation.

It is clear, therefore, that phenylglucuronide has not been satisfactorily described. We shall show that phenylglucuronide is normally a dihydrate unless dried at elevated temperatures. Its rate of hydrolysis by acid compared with that of phenylsulphuric acid has also been studied with a view to justifying a procedure used in this laboratory for separating phenols conjugated with sulphuric acid from those conjugated with glucuronic acid.

EXPERIMENTAL AND RESULTS

Phenylglucuronide

(*a*) *Isolation from urine.* The isolation of the glucuronide as the basic Pb salt has been described by Porteous & Williams (1949*a*). Phenol was fed at a dose level of 0·8 g. to rabbits (3 kg.), and from an 18 hr. urine the yield of crystalline glucuronide was 0·9–1·0 g./g. of phenol fed. To purify the glucuronide it was dissolved in absolute ethanol and the solution was filtered and evaporated to dryness *in vacuo*; the residue was recrystallized from the minimum of hot water. After drying *in vacuo* at room temperature the *dihydrate* of phenylglucuronide was obtained as long colourless needles, m.p. 161–162° (corr., decomp.) after sintering at 110–115°. It showed $[\alpha]_D^{17^\circ}$ – 78·5° (*c*, 2 in water). (Found: C, 47·5; H, 5·9; H_2O, 11·7; C_6H_5OH, 30·0%; equiv. by titration, 299. $C_{12}H_{14}O_7.2H_2O$ requires C, 47·1; H, 5·9; H_2O, 11·8; C_6H_5OH, 30·7%; equiv. 306.) After recrystallization from benzene-ethanol the compound retained its water. It is non-reducing and gives a strong Tollens naphthoresorcinol reaction.

On drying to constant weight at 105° (1·5–2 hr.) anhydrous phenylglucuronide as described by Masamune (1933) was obtained. This formed white needles, m.p. 161–162° (corr., decomp.) and $[\alpha]_D^{18^\circ}$ – 90·5° (*c*, 1·6 in water). (Found: equiv. 272. Calc. for $C_{12}H_{14}O_7$, equiv. 270.) The anhydrous compound dissolved less rapidly in water than the dihydrate.

It is probable that the optical rotations quoted by Neuberg & Niemann (1905) and Salkowski & Neuberg (1906) are for the dihydrate and that their analytical figures are for dried samples.

(*b*) *Benzylamine salt of phenylglucuronide.* The dihydrate (200 mg.) was dissolved in 20 ml. ethyl acetate containing 5% (v/v) absolute ethanol. Benzylamine (10 drops) was added until no further precipitation occurred. The *benzylamine salt* (200 mg.) was recrystallized from 95% ethanol and formed colourless needles, m.p. 207–208° (corr., decomp.), $[\alpha]_D^{25^\circ}$ – 62·3° (*c*, 2 in water). (Found: N, 3·5%. $C_{19}H_{23}O_7N$ requires N, 3·7%.) It is easily soluble in water, insoluble in cold but soluble in hot ethanol. It gives the Tollens reaction readily.

(*c*) *Absorption spectra.* The ultraviolet absorption spectra (determined with a Hilger E3 Quartz Spectrograph) of phenylglucuronide and its dihydrate were identical (the common curve is shown in Fig. 1). From the values of $E_{1\,cm.}^{1\%}$ for the anhydrous and hydrated forms at various wavelengths, the molecular weight of the hydrate could be calculated from that of the anhydrous form (mol. wt. 270). For the wavelengths 271, 268 and 259·5 mμ. the values 305, 311 and 309 respectively, were found (calc. mol. wt. 306), thus confirming that the compound was a dihydrate.

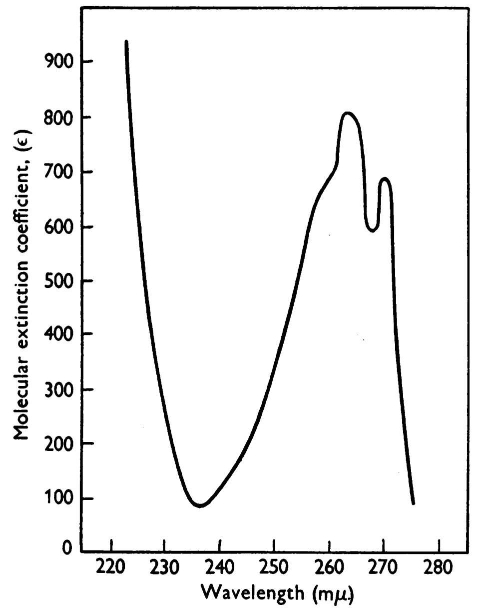

Fig. 1. Ultraviolet absorption spectrum of phenylglucuronide or its dihydrate in water. $\lambda_{max.}$ 271 and 264·5 mμ. ($\epsilon_{max.}$ 690 and about 810, respectively), and ~ 259·5 mμ. ($\epsilon_{max.}$ 710).

The spectra (Fig. 1) show bands with $\lambda_{max.}$ 271 and 264·5 mμ. ($\epsilon_{max.}$ 690 and about 810, respectively, and an inflexion at 259·5 mμ. with $\epsilon_{max.}$ 710. It is interesting to compare these values with those for phenol in ionizing and non-ionizing media. Phenylglucuronide (C_6H_5OR, where $R = C_6H_9O_6$) should be similar to un-ionized phenol ($R = H$). Phenol in pentane (non-ionizing medium) shows maxima at $\lambda_{max.}$ 270 and 277·5 mμ. ($\epsilon_{max.}$ 2000 in both cases) and a small band at 265 mμ. ($\epsilon_{max.}$ 1270; Klingstedt, 1923). In ionizing solvents phenol shows a single large band in this region of the ultraviolet; thus in water this band has $\lambda_{max.}$ 269·8 mμ. (Klingstedt, 1922) and in ethanol $\lambda_{max.}$ 273 mμ. (Morton & Stubbs, 1940). The spectrum of phenol in various solvents is further discussed by Stimson & Reuter (1945).

The rate of hydrolysis of phenylglucuronide by acid compared with that of phenylsulphuric acid

It has been observed on several occasions in this laboratory that glucuronides of phenols are not rapidly hydrolysed by dilute acids at the temperature of a boiling water bath, whereas ethereal sulphates of phenols are readily hydrolysed. We have made use of this observation for the separation of phenols conjugated with sulphuric acid from those conjugated with glucuronic acid (Garton & Williams, 1948, 1949; Porteous & Williams, 1949*b*; Smith & Williams, 1949). The present experiments were carried out to see how far this procedure was justified and could be made more discriminating.

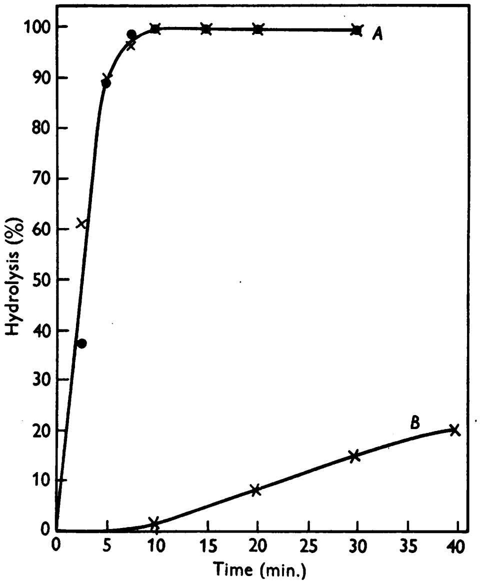

Fig. 2. Comparison of the rate of hydrolysis by N-HCl of phenylsulphuric acid with that of phenylglucuronide at 93–95°. *A*, potassium phenylsulphate, × 0·0048 M, ● 0·0017 M. *B*, phenylglucuronide dihydrate, 0·0005 M.

The potassium phenylsulphate used was prepared according to Burkhardt & Lapworth (1926). (Found: S, 15·4%. Calc. for $C_6H_5O_4SK$: S, 15·1%.)

Standard solutions of phenylglucuronide dihydrate (0·0005 M) and potassium phenylsulphate (0·0017 and 0·0048 M), made N with respect to HCl, were heated in a boiling water bath (temperature of solutions 93–95°). All solutions were raised to 93–95° before mixing. Samples of 5 ml. were withdrawn periodically and immediately neutralized with solid $NaHCO_3$. The mixture was diluted to a known volume with distilled water and its free phenol content determined colorimetrically with 2:6-dichloroquinonechloroimide as described by Porteous & Williams (1949*a*). The results (Fig. 2) show that, at this temperature, phenylsulphuric acid is completely hydrolysed in 10 min. whereas the proportion of phenylglucuronide hydrolysed is less than 2%. In previous papers we had used periods of 20–30 min. and, although less than 10% of the glucuronide is hydrolysed in this time, it appears that a 15 min. hydrolysis under our conditions would have given a sharper separation. In experiments in which the liberated sulphate was estimated, Sperber (1948) showed that phenylsulphuric acid was completely hydrolysed by approx. 0·25 N-HCl in 10 min. and resorcinylsulphuric acid in 15 min. Masamune (1933 studied the hydrolysis of phenylglucuronide by N-HCl at 100° (i.e. in boiling solution) and found 16% hydrolysis in 10 min. and 99% in 3·5 hr. Porteous & Williams (1949*a*) found that 10 N-H_2SO_4 was necessary to hydrolyse phenylglucuronide completely in 1 hr. at 100°.

SUMMARY

1. Phenylglucuronide has been prepared biosynthetically and characterized. It normally occurs as a dihydrate; its benzylamine salt has been described.

2. The ultraviolet absorption spectrum of the glucuronide in water has been determined and discussed in relation to that of phenol.

3. Phenylsulphuric acid is hydrolysed by acid at 93–95° at least 50 times as rapidly as is phenylglucuronide.

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