2. This method has been applied to the determination of catechols formed during the metabolism of fluoro-, chloro-, bromo-, iodo- and *o*-dichlorobenzene in the rabbit. Between 20 and 30 % of the monohalogenobenzenes metabolized are converted into the corresponding 4-halogenocatechols which are excreted mainly in conjugated forms for 2-3 days after dosing. Some 8 % of *o*-dichlorobenzene is dihydroxylated. The isomeric monochlorophenols and phenol are also oxidized to catechols to a small extent (0.5-4 %).

3. The halogenobenzenes also form small amounts

(about 2-3 %) of *p*-halogenophenols and traces of the *o*-phenols. These were detected mainly by paper chromatography.

4. The relation between mercapturic acid and *o*-dihydroxylation in these compounds is discussed, but no definite conclusions have been reached.

5. It is shown that the formation of catechols from the halogenobenzenes need not be contrary to the free radical hypothesis of biological hydroxylation, although other explanations are also feasible.

The expenses of this work were in part defrayed by a grant from the Medical Research Council.

REFERENCES

- Azouz, W. M., Parke, D. V. & Williams, R. T. (1952). *Biochem. J.* **50**, 702.
Baumann, E. & Schmitz, P. (1895). *Hoppe-Seyl. Z.* **20**, 586.
Bray, H. G., Humphris, B. G., Thorpe, W. V., White, K. & Wood, P. B. (1952). *Biochem. J.* **52**, 419.
Boyland, E. (1950). *Symp. biochem. Soc.* no. 5, 40.
Boyland, E. & Wiltshire, G. H. (1952). *Biochem. J.* **50**, xxxvii.
Callow, E. H. & Hele, T. S. (1926). *Biochem. J.* **20**, 598.
Corse, J. & Ingraham, L. L. (1951). *J. org. Chem.* **16**, 1345.
Fournau, E. & Druey, J. (1934). *C.R. Acad. Sci., Paris*, **199**, 870.
Garton, G. A. & Williams, R. T. (1949). *Biochem. J.* **45**, 158.
Goodwin, T. W. (1952). *Comparative Biochemistry of Carotenoids*. London: Chapman and Hall.
Gordon, S. & Schreyer, J. M. (1951). *Analyt. Chem.* **23**, 381.
Johnson, G. R. A., Stein, G. & Weiss, J. (1951). *J. chem. Soc.* p. 3275.
Parrod, J. (1948). *Bull. Soc. chim. Fr.* p. 761.
Peratoner, A. (1898). *Gazz. chim. ital.* **28**, I, 222.
Porteous, J. W. & Williams, R. T. (1949). *Biochem. J.* **44**, 56.
Radomski, J. L. & Davidow, B. (1952). *Fed. Proc.* **11**, 383.
Robinson, D., Smith, J. N. & Williams, R. T. (1951). *Biochem. J.* **50**, 228.
Rosenmund, K. W. & Kuhnhehn, W. (1923). *Ber. dtsh. chem. Ges.* **56**, 1268.
Smith, J. N. (1950). *Symp. Biochem. Soc.* no. 5, 15.
Smith, J. N., Spencer, B. & Williams, R. T. (1950). *Biochem. J.* **47**, 284.
Smith, J. N. & Williams, R. T. (1950). *Biochem. J.* **46**, 243.
Spencer, B. & Williams, R. T. (1950). *Biochem. J.* **47**, 279.
Stekol, J. A. (1936). *J. biol. Chem.* **113**, 279.
Weinland, R. F. & Döttinger, A. (1918). *Z. anorg. Chem.* **102**, 223.
Young, L. (1950). *Symp. biochem. Soc.* no. 5, 27.

Studies in Detoxication

52. THE APPARENT DISSOCIATION CONSTANTS OF SOME GLUCURONIDES, MERCAPTURIC ACIDS AND RELATED COMPOUNDS

BY D. ROBINSON, J. N. SMITH AND R. T. WILLIAMS

Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2

(Received 18 February 1953)

Quick (1932) noted that the conjugated products formed during detoxication processes in the body were highly acidic, and suggested that the purpose of conjugation may not primarily be a detoxication mechanism in the sense of rendering a toxic group inert, but a means of converting a weak acid into a strong acid which can be readily eliminated. That conjugated compounds such as glucuronides, hippuric acids, arylsulphuric acids are strongly acidic seems to be generally true but the ionization constants of only a few of these compounds have been measured (cf. Williams, 1947).

EXPERIMENTAL

Compounds. The glucuronides, mercapturic acids and phenylacetyl glycine used had been prepared biologically from time to time in this laboratory. Their melting points and rotations were checked with the literature. The hippuric acids and ornithuric acid used were synthetic.

Measurement of pK_a. The apparent dissociation constants of the acids at 22° were determined in the usual manner from the potentiometric titration curves. Measurements of pH were made with a Cambridge pH meter using a glass electrode. The compound (15-30 mg.), dissolved in freshly boiled distilled water to give an approximately 0.001M

solution, was titrated with 0.02N-NaOH, measuring the pH at 0.1-0.2 ml. intervals. pK_a was taken as the pH at which half the acid was neutralized. Salts were titrated with 0.02N-HCl.

The pK_a values of amphoteric compounds, such as the aminophenylglucuronides and quinolyglucuronides, were determined by titration with both acid and alkali. With most of the compounds the pK_a values of the acidic and basic groups were often not more than two pH units apart and it was not easy to distinguish between them. The basic pK_a values were, therefore, determined independently by a

Fig. 1. The ultraviolet-absorption spectrum of *p*-aminophenylglucuronide between 220 and 250 $m\mu$. in 0.1N-NaOH, 0.1N-HCl, and acetate buffers of various pH values.

spectroscopic method (cf. Morton & Lunn, 1952). The ultraviolet-absorption spectra of the aminophenylglucuronides, *p*-aminohippuric acid and quinolyglucuronides, vary with pH. This variation appears to depend on the presence or absence of a positively charged N. The absorption spectra of these compounds were, therefore, measured in 0.1N-HCl and 0.1N-NaOH and in buffers of known pH, using a Unicam S.P. 500 spectrophotometer. The pK_a of the basic group was then calculated from the relation

$$pK_a = pH - \log (\epsilon_a - \epsilon) / (\epsilon - \epsilon_b),$$

where ϵ is the molecular extinction coefficient at a given wavelength at a fixed pH, and ϵ_a and ϵ_b are the corresponding extinctions in acid and alkali. The relevant portions of the absorption curves for *p*-aminophenylglucuronide are shown in Fig. 1. The values of the spectroscopically determined $pK_{NH_3^+}$ given in Tables 2 and 3 are the averages of a number

of determinations at different pH values. Conjugation has little effect on the $pK_{NH_3^+}$ of the precursor (see Table 2). The pK values for thirty-one conjugated compounds are given in Tables 1-3.

Maximum values of λ and ϵ for the aminophenylglucuronides in acid and alkali are quoted in Table 4. The alkaline spectra of these glucuronides are very similar to the corresponding aminophenols (cf. Morton & Stubbs, 1940).

The optical rotations of the aminophenylglucuronides. The optical rotations of the aminophenylglucuronides in 0.1N-HCl were determined by Williams (1943). Bray, Clowes & Thorpe (1952a) have recently prepared these compounds and determined their rotations in water. It is well known that the rotations of amino acids vary with pH (cf. Neuberger, 1948), and it appears that the aminophenylglucuronides behave somewhat similarly. In acid solutions these glucuronides will occur as cations and in alkali as anions. Table 5 shows that the optical rotations of these compounds are more negative in alkali than in acid, especially those of the *o*- and *m*-isomers. The *p*-isomer, however, only shows very small differences, and Williams (1943) showed that this compound analysed as a monohydrate, whereas the *o*- and *m*-isomers were anhydrous. The sample of the *p*-isomer used in this work was newly prepared and had m.p. 215° (decomp.). (Found: C, 47.6; H, 5.5. Calc. for $C_{12}H_{15}O_7N \cdot H_2O$: C, 47.5; H, 5.6%.) On heating to 120° it lost no water, but on prolonged heating it darkened and showed signs of decomposition.

DISCUSSION

It is clear from Tables 1 and 2 that conjugated glucuronic acids are all fairly strong organic acids having pK_a values between 3 and 4. (Acetic and benzoic acids have pK_a values of 4.7 and 4.2.) It is to be expected, from structural considerations, that the carboxyl group of β -glucuronides would not be very different in acidity from that of glucuronic acid itself, which has a pK_a of 2.93. At the pH of blood (7.4), glucuronides are, therefore, practically completely ionized and, in general, one would expect them to be readily excreted. Furthermore, they are usually stronger acids than their precursors even though these may be carboxylic acids such as veratric acid (pK_a , 4.44). Veratroylglucuronide has pK_a 3.47 (Table 1). Benzoic acid (pK_a , 4.2) forms benzoylglucuronide for which Quick (1932) quotes a pK_a value of about 2.52, which is probably somewhat low. Glucuronides may be formed from primary, secondary and tertiary alcohols in which the pK_a of the hydroxyl group is in the range 12-19, from phenols, pK_a range 5-11, and from some carboxylic acids, pK_a range 3-5. If the purpose of the conjugation is to increase acid strength so as to produce a highly ionizable compound which would be rapidly excreted, it may be expected that, other things being equal, a compound with a pK_a of about 3 or less, i.e. less than that of a glucuronide, would not be conjugated. This is true to some extent since *o*-nitrobenzoic acid (pK_a , 2.21) and *o*-chlorobenzoic acid (pK_a , 2.89) are excreted largely unchanged by rabbits (Bray, Thorpe & Wood, 1949; Bray, Clowes,

Table 1. *Apparent dissociation constants of biosynthetic glucuronides*

(The pK_a values for the precursors are taken from the literature. Alcohols are very weak acids having values between 16 and 19 on an arbitrary pK_a scale, e.g. menthol has a pK_a of 19 (cf. Hammett, 1940).)

β -Glucuronide (Glucuronic acid)	pK _a (2.93)	Precursor —	pK _a —
Ether type (R.O.C ₆ H ₅ O ₆)			
Phenyl	3.42	Phenol	10
<i>p</i> -Bromophenyl	3.02	<i>p</i> -Bromophenol	9.8
<i>p</i> -Iodophenyl	3.28	<i>p</i> -Iodophenol	9.5
<i>p</i> -Cyanophenyl	3.22	<i>p</i> -Cyanophenol	8.2
<i>p</i> -Diphenyl	3.10	<i>p</i> -Hydroxydiphenyl	—
2:4-Dimethylpentyl-3-	3.89	2:4-Dimethylpentane-3-ol	Approx. 18
(+)- <i>iso</i> Menthyl	3.70	(+)- <i>iso</i> Menthol	Approx. 18
1-Hydroxyxanthone-7-	3.94	1:7-Dihydroxyxanthone	—
(Euxanthic acid)		(Euxanthone)	
Ester type (R'.CO.O.C ₆ H ₅ O ₆)			
Veratroyl	3.47	Veratric acid	4.44
2-Ethylhexanoyl	3.08	2-Ethylhexanoic acid	—
Diethylacetyl	3.37	Diethylacetic acid	4.7

Table 2. *Apparent dissociation constants of biosynthetic amphoteric glucuronides*

(Values in parentheses were determined spectroscopically. Values for the precursors are taken from the literature. The glucuronides of the hydroxyquinolines are described by Smith (1953). The 2- and 4-quinolones do not conjugate directly but are first hydroxylated, so that *x* may be 3, 5, 6, 7 or 8.)

β -Glucuronide	pK _{COOH}	pK _{NH₃⁺}	Precursor	pK _{OH}	pK _{NH₃⁺}
<i>o</i> -Aminophenyl	3.30	4.78	<i>o</i> -Aminophenol	9.72	4.72
<i>m</i> -Aminophenyl	2.97	4.44	<i>m</i> -Aminophenol	9.89	4.17
		(4.48)			
<i>p</i> -Aminophenyl	3.1	5.3	<i>p</i> -Aminophenol	10.30	5.49
		(5.2)			
Quinolyl-3-	3.0	3.95	3-Quinololinol	—	—
Quinolyl-5-	3.1	4.91	5-Quinololinol	—	—
Quinolyl-6-	3.0	5.00	6-Quinololinol	—	—
Quinolyl-7-	3.0	5.40	7-Quinololinol	—	—
		(5.25)			
Quinolyl-8-	3.01	4.85	8-Quinololinol	9.45*	5.03*
		(4.4)			
2-Quinolone- <i>x</i> -	3.07	3.75	2-Quinolone	—	—
4-Quinolone- <i>x</i> -	3.15	3.7	4-Quinolone	—	—

* Albert & Magrath (1947).

Table 3. *Apparent dissociation constants of mercapturic, hippuric and ornithuric acids*

(Values for the precursors and acetylglycine are taken from the literature. The pK_{NH₃⁺} value 3.19 for *p*-aminohippuric acid was determined spectroscopically.)

Compound	pK _a	Precursor	pK _a
L-Phenylmercapturic acid	3.60	Benzene	—
L- <i>p</i> -Chlorophenylmercapturic acid	3.77	Chlorobenzene	—
L- <i>p</i> -Bromophenylmercapturic acid	3.81	Bromobenzene	—
L- <i>p</i> -Iodophenylmercapturic acid	3.78	Iodobenzene	—
Acetylglycine	3.6	—	—
Hippuric acid	3.70	Benzoic acid	4.2
<i>o</i> -Chlorohippuric acid	3.79	<i>o</i> -Chlorobenzoic acid	2.89
<i>o</i> -Hydroxyhippuric acid (Salicyluric acid)	3.62	Salicylic acid	3.23
<i>p</i> -Aminohippuric	4.03	<i>p</i> -Aminobenzoic acid	4.86
	(pK _{NH₃⁺} , 3.03)		(pK _{NH₃⁺} , 2.32)
	3.19		
Phenylacetylglycine (Phenaceturic acid)	3.67	Phenylacetic acid	4.25
DL- <i>NN'</i> -Dibenzoylornithine (DL-Ornithuric acid)	3.70	Benzoic acid	4.2

Table 4. *Maximum values of λ and ϵ for the aminophenylglucuronides*

Isomer	In 0.1 N-HCl		In 0.1 N-NaOH			
	$\lambda_{\max.}$ (m μ .)	$\epsilon_{\max.}$	$\lambda_{\max.}$ (m μ .)	$\epsilon_{\max.}$	$\lambda_{\max.}$ (m μ .)	$\epsilon_{\max.}$
Ortho	270	890	230	8100	280	2150
Meta	265	1070	232	8600	280	2250
Para	270	750	232.5	9800	290	1800

Thorpe, White & Wood, 1952b). Furthermore, *m*- and *p*-nitrobenzoic acids (pK_a 3.46 and 3.4) are also excreted mainly unchanged (Bray *et al.* 1949). However, some strong acids are conjugated (see below).

Table 5. *The optical rotations of the aminophenylglucuronides*

Isomer	[α] _D ²⁰ in		
	0.1 N-HCl (c, 1)	0.1 N-NaOH (c, 1)	Water (c, 0.5)
Ortho	-77.5° (-76.1)*	-94°	-77° (-87)†
Meta	-94 (-94)*	-106	-97 (-100)†
Para	-84 (-82.7)*	-88	-86 (-93)†

* Williams (1943).

† Bray *et al.* (1952a).

The mercapturic acids quoted in Table 3 all have pK_a about 3.7 and are derived from non-acidic hydrocarbons. The hippuric acids (Table 3) also have pK_a values in the region of 3.7, close, as would be expected, to that of acetylglycine (pK_a , 3.6). Ornithuric acid, which appears to be formed only in birds, also has a pK_a of 3.7. Thus, all the thirty-one conjugated compounds recorded in this paper have pK_a values between 3 and 4. Conjugated sulphuric acids (ethereal sulphates) are even stronger than this, for Burkhardt, Ford & Singleton (1936) have shown that potassium phenylsulphate and potassium *p*-nitrophenylsulphate are salts of strong acids, since they show, with hydrochloric acid, no buffering action detectable by indicators, whereas potassium sulphate shows marked buffering action under the same conditions. Furthermore, in some animals, pyridine (pK_a , 5.21) and quinoline (pK_a , 4.94) are methylated and excreted as the highly ionized methyl-pyridinium and methyl-quinolinium hydroxides which are known to be strong bases, although no pK_a values appear to have been recorded. Furthermore, the weak hydrocyanic acid (pK_a , 8.88) is converted in animals into the much stronger thiocyanic acid.

o-Chlorobenzoic acid (pK_a , 2.89) is excreted largely unchanged by rabbits, but a small amount of it forms an ester glucuronide which presumably would have a pK_a of just over 3. It also conjugates to a slight extent with glycine (Bray *et al.* 1949) to

form *o*-chlorhippuric acid (pK_a , 3.79). Thus, both glucuronic acid and glycine conjugations produce acids weaker than the precursor. It is possible that in order to become conjugated a compound must enter a cell, and owing to its low pK_a , *o*-chlorobenzoic acid only does so to a small extent. Thus its conjugation is low. In the case of salicylic acid (pK_a , 3.23) the formation of salicyluric acid (pK_a , 3.62), or of salicylglucuronides (which would probably have pK_a values about 3.3-3.5) or gentisic acid (pK_a , 3.32), hardly makes any difference in acidity. However, despite its relatively high acidity, salicylic acid appears to be appreciably oxidized and conjugated in some species. According to Bray, Ryman & Thorpe (1948) nearly 30% may form glucuronides and at least 5% may be oxidized to gentisic acid in the rabbit. In man, it is mainly converted to salicyluric acid (Kapp & Coburn, 1942); some 25% is converted to glucuronides and 4-8% oxidized to gentisic acid. According to Alpen, Mandel, Rodwell & Smith (1951) (cf. also Schayer, 1950) the amounts of metabolites of salicylic acid excreted by man are variable. They give salicylic acid 10-85, salicyluric acid 0-50, gentisic acid up to 1 and salicylglucuronides 15-40% of the dose. Salicylic acid, however, appears to penetrate into all the tissues of the body (Goodman & Gilman, 1941; cf. also Gross & Greenberg, 1948). These facts suggest that compounds may conjugate with glucuronic acid (or glycine in the case of carboxylic acids) irrespective of their pK_a if they can penetrate cells.

According to Höber (1946) a conjugation mechanism may have two effects. In the first place, the toxic substances, which are often weak electrolytes and lipid-soluble and thus are apt to enter cells, are converted into strong electrolytes and this results in an incapacity to enter cells. The second effect is the formation of a hydrophobic-hydrophilic compound which can be eliminated by way of the kidney. Sperber (1948) has shown that a number of glucuronides and ethereal sulphates and hippuric acid are readily excreted by the tubules in the chicken. The present results show that all conjugates are strong electrolytes, and it is also known that they possess the polar-non-polar or so-called organophilic-hydrophilic structure. Many studies in the past have suggested that the polarity of a molecule largely determined whether or not a compound was

actively excreted by the renal tubules. The relation between pK_a and excretion has been studied by Fisher, Troast, Waterhouse & Shannon (1943) who found that although in many cases the polarity of the compound was important, several weakly polar compounds, e.g. sulphanilylsulphanilamide, were actively secreted whilst some strongly polar molecules, e.g. sulphanilic acid, were not; they concluded that structure and possibly other factors, as well as pK_a, were involved.

The present work shows that the majority of conjugation processes result in the production of relatively strong electrolytes, which are easily excreted, from weaker ones. Organic compounds which are stronger electrolytes than their possible corresponding conjugates tend to be excreted largely unchanged, unless they can penetrate cells. In the latter event conjugation appears to take place irrespective of whether the pK_a of the precursor is greater or less than that of the conjugate formed.

The relation of pK_a to type of conjugation may be summarized: hippuric acid formation, when it occurs, appears to be limited to carboxylic acids where the pK_a range is 2–5, ethereal sulphate formation occurs usually with phenols where the

pK_a range is 6–11; glucuronic acid conjugation, however, when it occurs, appears to take place irrespective of pK_a from 2–20.

SUMMARY

1. The apparent dissociation constants of twenty-one biosynthetic β -glucuronides have been measured. The pK_a values were all between 3 and 4, and close to that of glucuronic acid (2.93).

2. *p*-Chloro-, *p*-bromo- and *p*-iodo-phenylmercapturic acids had pK_a values about 3.8, near to that of phenylmercapturic acid (3.6). Ornithuric acid had pK_a 3.7.

3. Five hippuric acids had pK_a about 3.7, close to that of acetylglutamine (3.6).

4. The above results are discussed and it is shown that most conjugated compounds are stronger electrolytes than their precursors. Some exceptions to this rule are explained.

5. The absorption spectra and optical rotations of the aminophenylglucuronides have been determined and discussed.

The expenses of this work were in part defrayed by a grant from the Medical Research Council.

REFERENCES

- Albert, A. & Magrath, D. (1947). *Biochem. J.* **41**, 534.
- Alpen, E. L., Mandel, H. G., Rodwell, V. W. & Smith, P. K. (1951). *J. Pharmacol.* **102**, 150.
- Bray, H. G., Clowes, R. C. & Thorpe, W. V. (1952a). *Biochem. J.* **51**, 70.
- Bray, H. G., Clowes, R. C., Thorpe, W. V., White, K. & Wood, P. B. (1952b). *Biochem. J.* **50**, 583.
- Bray, H. G., Ryman, B. E. & Thorpe, W. V. (1948). *Biochem. J.* **43**, 561.
- Bray, H. G., Thorpe, W. V. & Wood, P. B. (1949). *Biochem. J.* **44**, 39.
- Burkhardt, G. N., Ford, W. G. K. & Singleton, E. (1936). *J. chem. Soc.* p. 17.
- Fisher, S. H., Troast, L., Waterhouse, A. & Shannon, J. A. (1943). *J. Pharmacol.* **79**, 389.
- Goodman, L. & Gilman, A. (1941). *The Pharmacological Basis of Therapeutics*, p. 229. New York: Macmillan.
- Gross, M. & Greenberg, L. A. (1948). *The Salicylates*, pp. 19–71. New Haven: Hillhouse Press.
- Hammett, L. P. (1940). *Physical Organic Chemistry*, p. 50. New York: McGraw-Hill.
- Höber, R. (1946). *Physical Chemistry of Cells and Tissues*, p. 571. London: Churchill.
- Kapp, E. M. & Coburn, A. F. (1942). *J. biol. Chem.* **145**, 549.
- Morton, R. A. & Lunn, A. K. (1952). *Analyst*, **77**, 718.
- Morton, R. A. & Stubbs, A. L. (1940). *J. chem. Soc.* p. 1349.
- Neuberger, A. (1948). *Advanc. Protein Chem.* **4**, 298.
- Quick, A. J. (1932). *J. biol. Chem.* **97**, 417.
- Schayer, R. W. (1950). *Arch. Biochem.* **28**, 371.
- Smith, J. N. (1953). *Biochem. J.* **55**, 156.
- Sperber, I. (1948). *K. LandtbrHogsk. Ann. (Ann. Roy. Agric. Coll., Sweden)*, **15**, 317.
- Williams, R. T. (1943). *Biochem. J.* **37**, 329.
- Williams, R. T. (1947). *Detoxication Mechanisms*, p. 247. London: Chapman and Hall.