CLXIII. THE BIOCHEMISTRY AND PHYSIOLOGY OF GLUCURONIC ACID.

III. THE STRUCTURE OF BENZOYLGLUCURONIC ACID.

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MUCH attention has been paid to the detoxication of aromatic acids of the benzoic acid type in the animal body, but all the earlier workers failed to recognise the part played by glucuronic acid in this process, and attention was centred on the problem of the conjugation of these acids with glycine and their subsequent excretion as hippuric acids. There is now no doubt that glucuronic acid plays a very important rôle in the detoxication of aromatic acids, especially when, after the administration of large doses of the toxic acid, the preformed glycine of the body has become exhausted. The detoxication of compounds of the benzoic acid type has been the subject of detailed study by Quick [1926, 1; 1928, 1, 2; 1931; 1932, 1, 2, 3, 4].

The various conjugated glucuronic acids may be divided into three types.

(1) Conjugated glucuronic acids of the glycoside-“ether” type, such as bornylglucuronide, I [*cf.* Pryde and Williams, 1933], in which the non-sugar residue is attached to the reducing group of glucuronic acid by means of a true glycoside link:

Glucuronides of this type are non-reducing.

(2) Conjugated glucuronic acids of the glycoside-“ester” type, such as benzoylglucuronide, II (benzoylglucuronic acid), in which the benzoyl radical is attached to the reducing carbon of glucuronic acid. This linkage is unstable to hydrolytic reagents and these glucuronides are reducing.

(3) Conjugated glucuronic acids containing two glucuronic acid molecules, one attached to the non-sugar residue by a glycoside-“ether” linkage as in

type (1) and the other attached by an "ester" link as in type (2); the *p*-hydroxybenzoic acid-diglyceronic acid, III, described by Quick [1932, 3] is an example of this type:

Benzoylglucuronic acid was first isolated as the sodium and strychnine salts by Magnus-Levy [1907], and he assigned to it the constitution of a 1-benzoylglucuronide, IV. The free acid was, however, prepared for the first time by Quick [1926, 2] from the urine of dogs fed with benzoic acid, but no analytical data other than titrations were given. Owing to the fact that this acid is very unstable to hydrolytic reagents, especially alkali, it reduces Fehling's solution and other alkaline sugar reagents. Quick, therefore, supposed it to be a 2-benzoylglucuronic acid, V, which would possess a free reducing (potential) aldehyde group. Various considerations led us to doubt the correctness of this view and to support the original structure suggested by Magnus-Levy (using the new amylene-oxidic ring instead of the original butylene-oxidic ring).

In the first place, on scrutinising the published data concerning glycosides containing a 1-benzoyl radical, it is found that all these glycoside-"esters" reduce Fehling's solution, owing to the ease of hydrolysis of the benzoyl group in the presence of alkali. The data are tabulated below (Table I).

Table I. *Glycosides containing a 1-benzoyl radical, which reduce Fehling's solution.*

Compound	Authority
1-Benzoyl-4 : 6-benzylideneglucose	Zervas [1931]*
1 : 2 : 3-Tribenzoyl-4 : 6-benzylideneglucose	Brigl and Grüner [1932]
1 : 2 : 3-Tribenzoylglucose	"
1 : 2 : 3-Tribenzoyldiacetylglucose	"
1 : 2 : 3 : 4-Tetrabenzoylglucose	"
1 : 2 : 3 : 4-Tetrabenzoylmonoacetylglucose	"
1 : 3 : 4 : 5-Tetrabenzoylfructose	Brigl and Schinle [1933]
1 : 3 : 4 : 5 : 6-Pentabenzoylfructose	"

* The reducing properties of this compound were not mentioned by Zervas but it was prepared by us by Zervas's method and found to be reducing.

It is also found that certain nitrogenous glycosides, such as the amino-acid- and the amino-acid ester-glycosides [Maurer and Schiedt, 1932] and glucosidureides [Haring and Johnson, 1933] reduce Fehling's solution owing to hydrolysis in the presence of alkali.

Secondly, an examination of the optical rotations of β -*d*-glucosides and the corresponding β -*d*-glucuronides shows these to be roughly of the same order. This observation accords with the expectation that the change of $-\text{CH}_2\text{OH}$ to $-\text{COOH}$ should have but a small influence on the rotation. Table II gives all the data available on this point and it will be noted that benzoylglucuronic acid and 1-benzoyl- β -*d*-glucoside [Zervas, 1931] fall in line with the others, thus giving further evidence for the 1-benzoyl-structure of benzoylglucuronic acid.

Table II. *Rotations and melting-points of d-glucosides and corresponding d-glucuronides.*

Compound	<i>d</i> -Glucoside				<i>d</i> -Glucuronide		
	$[\alpha]_D$	Solvent	M.P.		$[\alpha]_D$	Solvent	M.P.
(1) β -Benzoyl	-26.8°	Water	193°	(8)	-25.3°	Water	183°
(2) β -Phenyl	-71.7	Water	176	(9)	-82	Water	151
(3) β - <i>d</i> -Bornyl	-42.4	Alcohol	135	(10)	-37*	Water	174
(4) β - <i>l</i> -Bornyl	-60.1	Alcohol	—	(11)	-66.6	Water	—
(5) β - <i>l</i> -Menthyl	-93.6	Alcohol	76	(12)	-104.6	Alcohol	110
(6) α - <i>l</i> -Menthyl	+64	Alcohol	160	(13)	+52	Alcohol	130
(6) β -Phloroglucinyll	-74.8	Water	239	(14)	-80.8†	Water	—
(7) Urea	-23.4	Water	207	(15)	-21.2‡	Water	—

* Rotation of sodium salt.

† Rotation of potassium salt.

‡ Calculated from rotation of barium salt.

(1) [Zervas, 1931]. (2) [Fischer and Mechel, 1916]. (3) [Fischer and Raske, 1909]. (4) [Hämäläinen, 1913]. (5) [Fischer and Bergmann, 1917]. (6) [Fischer and Strauss, 1912]. (7) [Hynd, 1926; Fischer, 1914]. (8) This investigation. (9) [Salkowski and Neuberg, 1906]. (10) [Magnus-Levy, 1906]. (11) [Pryde and Williams, unpublished results]. (12) [H. Fischer, 1911]. (13) [Bergmann and Wolff, 1923]. (14) [Sera, 1914]. (15) [Neuberg and Niemann, 1905].

Quick [1926, 2] studied polarimetrically the action of NaCN on benzoylglucuronic acid (*cf.* Fig. 1) and considered that the observed changes were due

Fig. 1. The action of KCN and NaCN on benzoylglucuronic acid. ○ Quick [1926, 2] for NaCN; × Present investigation with KCN.

to mutarotation (*i.e.* of a 2-benzoylglucuronide) and the formation of a cyanohydrin of benzoylglucuronic acid, *B* (p. 1213). We have obtained similar results using KCN, but owing to the instability of the benzoyl group in the presence of

cyanide, a quite different interpretation is necessary. The alkalinity of the cyanide causes hydrolytic fission of the benzoyl radical and mutarotation and formation of the cyanohydrin of glucuronic acid, *A* (not benzoylglucuronic acid) follows. This view is supported by the fact that free benzoic acid was isolated when a solution of benzoylglucuronic acid and KCN was allowed to stand for 24 hours, and benzoylglucuronic acid does not react with HCN without splitting off benzoic acid. Since more than one molecule of cyanide [cf. Quick, 1926, 2] is required to react completely with one molecule of benzoylglucuronic acid, it is evident that some cyanide is used in hydrolysis.

Attempts to methylate benzoylglucuronic acid with Ag_2O and MeI, or with diazomethane failed, since the compound was hydrolysed during methylation and the benzoyl group was split off and formed methyl benzoate.

Improvements were effected in Quick's method [1926, 2] of preparing benzoylglucuronic acid, and a purer specimen of the acid was obtained, M.P. 183° (Quick quotes M.P. $170\text{--}172^\circ$).

EXPERIMENTAL.

The preparation and purification of benzoylglucuronic acid.

Benzoylglucuronic acid was prepared by Magnus-Levy [1907] as the strychnine and sodium salts from the urine of a sheep fed on sodium benzoate, while Quick [1926, 2] prepared the free acid from the urine of dogs similarly fed. In the present work 5 g. of benzoic acid were fed daily to each of several small dogs, the acid being contained in small gelatin capsules which were given with meat. The urines were collected every 24 hours and preserved with toluene. The urines were acid in reaction (p_{H} 3–4) and were laevorotatory. They were worked up every 3 days as follows. All reagents were kept cold in the refrigerator. The urine, after addition of a little acetic acid, was precipitated exactly with normal lead acetate and filtered. The filtrate was brought to p_{H} 6–7 with cold dilute ammonia and then precipitated with ice-cold saturated basic lead acetate. The precipitate was filtered immediately on a large Büchner funnel under suction and washed several times with ice-water. The precipitate was made into a thin paste with ice-water and decomposed with H_2S , and the lead sulphide filtered off through Seitz "Brilliant" asbestos. The filtrate was again treated with H_2S

to ensure complete removal of lead, which interferes with the successful crystallisation of benzoylglucuronic acid. The clear filtrate was aerated to remove H_2S and concentrated *in vacuo* until crystallisation of hippuric acid set in. The solution was then allowed to stand and the hippuric acid filtered off. At this point the filtrate was shaken with an equal volume of chloroform. The chloroform layer became red owing to extraction of pigments and also dissolved any free benzoic acid (which may be separated on evaporation of the chloroform). After separation of the chloroform layer, the aqueous layer generally deposited crystals immediately, and the crystallisation was completed in the refrigerator. The crude benzoylglucuronic acid was filtered off and recrystallised from a little hot water (decolorising with norite charcoal) and dried in a desiccator over CaCl_2 . The dried acid still contained hippuric acid and possessed a slight yellow colour. It was now pulverised (5 g.) and shaken up with ether (200 cc.). The ether was renewed three times in 24 hours; each time the acid was shaken with ether and allowed to stand until the ether was renewed. The product still contained traces of hippuric acid and was dissolved in a little methyl alcohol and the solution decolorised with norite. The filtered methyl alcohol solution was then precipitated with 20 times its volume of dry ether and the whole allowed to stand in the refrigerator to complete the crystallisation of the precipitated benzoylglucuronic acid, which crystallised gradually but completely from the solution in aggregates of short, stout, radiating needles, colourless and free from traces of hippuric acid which was retained in the ether. The ether was decanted and the crystals dried.

They had M.P. 183° ; $[\alpha]_{\text{D}}^{21} - 29.9^\circ$ in water ($c = 1.639$); $[\alpha]_{\text{D}} - 25.3^\circ$; yield 5 g. from 36 g. benzoic acid fed. The acid is soluble in methyl and ethyl alcohols and water, slightly soluble in ethyl acetate and insoluble in ether and chloroform. Found, C, 52.2; H, 4.85 %; $\text{C}_{13}\text{H}_{14}\text{O}_8$ requires C, 52.3; H, 4.7 %.

The action of KCN on benzoylglucuronic acid.

0.25 g. of benzoylglucuronic acid was dissolved in an aqueous solution of potassium cyanide. The solution was clear initially, but after about 1 min. became opalescent and then cleared up again. The whole was allowed to stand with an equal volume of chloroform in a corked flask for 24 hours at room temperature. A similar solution was made up omitting the cyanide. The chloroform layer was then separated and evaporated at low temperature *in vacuo*. A crystalline residue was obtained in the case where KCN was added but no residue was obtained in the other case. The crystalline residue consisted of typical platelets of benzoic acid (M.P. 117° on recrystallisation from ether) and gave all the qualitative tests for this acid. It is probable that the greater proportion of the benzoic acid was converted into potassium benzoate. Similar experiments were made using NaHCO_3 instead of KCN but no free benzoic acid was obtained, since it was probably converted entirely into sodium benzoate.

The action of KCN was also followed polarimetrically; 0.1312 g. of benzoylglucuronic acid was dissolved in 10 cc. of water, and 7 cc. of the solution were mixed with 2 cc. of a solution of KCN (0.1405 g. in 10 cc.) and the change in rotation observed polarimetrically. The results are given in Fig. 1, which also gives the data of Quick [1926, 2] for the action of NaCN.

SUMMARY.

Conjugated glucuronic acids can be divided into three types; the glycoside-“ether” type, the glycoside-“ester” type and a third type which is a combination of the first and second types. Benzoylglucuronic acid (a glycoside-“ester”

derivative) has been prepared in a state of purity, and full analytical data are recorded for the first time. Like all glycosides containing a 1-benzoyl radical, benzoylglucuronic acid reduces sugar reagents owing to the ease of hydrolysis of the benzoyl radical. A study of the action of KCN on benzoylglucuronic acid, and certain other theoretical considerations, suggest that it is a 1-benzoyl derivative of glucuronic acid, and not a 2-benzoyl derivative. Attempts to methylate it with methyl iodide and silver oxide and with diazomethane failed since the benzoyl group is very unstable and is split off as methyl benzoate.

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