58. STUDIES IN DETOXICATION

10. THE CONJUGATION AND OXIDATION OF *p*-HYDROXYBENZENESULPHONAMIDE IN THE RABBIT. THE CHARACTERIZATION OF *p*-SULPHONAMIDOPHENYLGLUCURONIDE

BY H. G. SAMMONS,[1] JEAN SHELSWELL[1] AND R. TECWYN WILLIAMS

From the Physiology Department, Medical School, Hospitals Centre, Birmingham, 15

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INVESTIGATIONS in progress in this Laboratory on the fate of sulphonamide drugs in the animal body indicate that these are oxidized to a small extent with the production of phenolic substances which may be excreted in conjugation with sulphuric and glucuronic acids [cf. Shelswell & Williams, 1940; Thorpe & Williams, 1940; 1941; Thorpe *et al.* 1941]. In addition to this Scudi [1940] has isolated from the urine of dogs treated with sulphapyridine, the conjugated glucuronide of a hydroxysulphapyridine. It therefore became necessary to study the fate of an aryl sulphonamide containing a phenolic hydroxyl group, since such a study might be of some help in identifying the phenolic substances occurring in urine after the ingestion of sulphanilamide and related drugs. *p*-Hydroxybenzenesulphonamide (hereafter called phenol-*p*-sulphonamide) was selected as it was easily prepared and was also the simplest compound of the type required for study. No previous study has been made of its fate in the animal body, but Buttle *et al.* [1936], among others, have shown that it is therapeutically inactive.

The conjugation and oxidation of phenolsulphonamide

The present work shows that this sulphonamide when fed to rabbits is mainly excreted in the urine conjugated with glucuronic and sulphuric acids; in addition to this a very small portion is oxidized to a catechol derivative, possibly catechol-sulphonamide. No free phenolsulphonamide was detected in the urine, since ether extraction of the urine as such (faintly alkaline in reaction) and after acidification gave no trace of the sulphonamide. After hydrolysis with HCl, however, phenolsulphonamide together with the catechol compound could easily be extracted with ether.

The amount of phenolsulphonamide excreted conjugated with sulphuric acid depends upon the dose fed, the percentage conjugation falling with increasing dose (see Table 1). At the smallest dose level used (100 mg./kg.) the sulphate conjugation is more than 50 % of the dose, falling to less than 30 % at the highest level (750 mg./kg.). This behaviour recalls that of phenol, whose sulphate conjugation at various dose levels was studied by one of us [Williams, 1938]. Baumann & Herter [1877–8] have shown that the corresponding acid, phenol-*p*-sulphonic acid, produced no increase in ethereal sulphate excretion in the dog.

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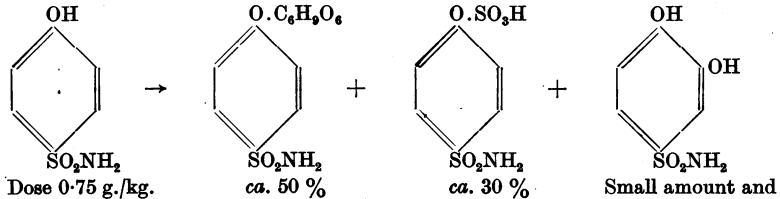
Table 2 shows that this is also the case for the rabbit. In addition to phenol-*p*-sulphonic acid (Na salt, B.D.H.) other phenolic sulphonic acids were also fed, namely, 1-hydroxy-2-amino- (B.D.H.), 1-amino-2-hydroxy- [Friedländer, 1911] and 1-amino-3-hydroxy-benzene-4-sulphonic [Friedländer, 1896] acids. None of these acids caused the formation of ethereal sulphate in the rabbit (see Table 2), although they possess a phenolic OH group. A similar result was obtained by Williams [1938] with salicylic acid. Neither was the formation of conjugated glucuronic acid detected (by the naphthoresorcinol reaction) after feeding these acids. Other forms of conjugation were not tested for, as they were very unlikely to occur with the above compounds. It seems very probable that these sulphonic acids are excreted unchanged by the rabbit. Table 2 also includes toluene-*p*-sulphonamide, which causes no increase in ethereal sulphate formation in the rabbit. This compound is of interest since Flaschenträger *et al.* [1934] have shown that about 31 % of it is oxidized to *p*-sulphonamidobenzoic acid in the dog.

Judging from the maximum amount of barium sulphonamidophenylglucuronate that could be isolated from rabbit urine after administration of the sulphonamide, some 50 % is excreted conjugated with glucuronic acid, the dose being 0·75 g./kg. Therefore when the dose is 0·75 g./kg., over 80 % of the phenolsulphonamide can be accounted for by the conjugated glucuronide and ethereal sulphate excreted in the urine.

It was found that crude specimens of the above barium salt gave a green colour with $FeCl_3$ in aqueous solution. This colour was not due to free phenolsulphonamide since the latter gives a feeble purple colour with $FeCl_3$ which is discharged by acid or alkali. The alcoholic filtrates from the preparation of the barium salt (see experimental section) were evaporated to a syrup. The syrup was water-soluble and reduced ammoniacal silver nitrate but not Fehling's solution. Its aqueous solution gave an intense green colour with $FeCl_3$ which turned to blue-green, purple and then red on making the solution progressively more alkaline with Na_2CO_3. Acid discharged the colour. These reactions are similar to those of catechol. Further investigation showed that the syrup was mainly *p*-sulphonamidophenylglucuronide containing a small amount of a catechol derivative which was responsible for the $FeCl_3$ test. This catechol compound was excreted in conjugation since the urine itself gave no $FeCl_3$ reaction nor could a $FeCl_3$ reaction be obtained on ether extracts of the urine at acid or faintly alkaline reactions. The substance could be extracted together with phenolsulphonamide from acid-hydrolysed urines. Several attempts were made to isolate and identify this compound, but owing to the smallness of the amount present, the attempts have so far been fruitless. Such attempts, however, are being continued in conjunction with other work of a similar nature. The very characteristic $FeCl_3$ test and the reduction of ammoniacal $AgNO_3$ leave no doubt in our minds that a catechol derivative is present.

The fate of phenol-*p*-sulphonamide in the rabbit can therefore be summarized as follows:

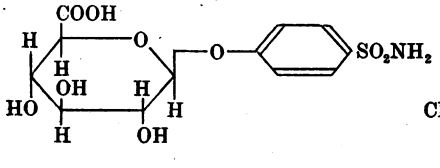


Dose 0·75 g./kg. *ca.* 50 % *ca.* 30 % Small amount and excreted conjugated

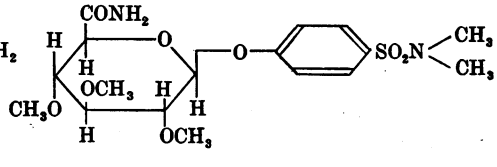
The formation of the above catechol derivative is an example of biological oxidation of the benzene ring in the *o*-position to an existing —OH. Baumann & Preusse [1879] have shown that this *ortho* oxidation takes place during the oxidation of phenol in the dog, since they found small amounts of catechol and quinol in the urine after larger doses of phenol. Biological oxidation can also take place *ortho* to an —NH_2, since *o*-aminophenols are produced in animals from a number of aromatic amino-compounds, e.g. formanilide [Kleine, 1896–7], acetanilide and *o*-acetotoluidide [Jaffe & Hilbert, 1888] and dimethylaniline [Horn, 1936]. There is also the case of β-naphthylamine which is oxidized in rabbits to β-amino-α-naphthol [Wiley, 1938]. A similar oxidation is possible with sulphanilamide [cf. Thorpe *et al.* 1941], since the formation of a catechol derivative from phenolsulphonamide suggests that biological oxidation can take place not only in a position *ortho* to the —OH group but also *meta* to the sulphonamide group.

The characterization of p-*sulphonamidophenylglucuronide*

Removal of barium from barium sulphonamidophenylglucuronate gave the free conjugated glucuronic acid as a brown uncrystallizable syrup. Many phenolic glucuronides behave in this manner and they have often been characterized as barium salts. These salts, however, are usually amorphous and difficult to purify. We therefore subjected the above barium salt to methylation with methyl sulphate and alkali followed by methyl iodide and silver oxide. In this manner the *methyl ester* of *trimethyl* p-*sulphondimethylamidophenylglucuronide* was obtained as a syrup (the sulphonamide group being methylated as well as the sugar residue). Treatment of this ester with methyl alcoholic ammonia gave a crystalline *amide* (II) whereby the glucuronide (I) could be characterized:



(I)



(II)

Pryde & Williams [1933] have shown that, in the case of biosynthetic bornylglucuronide, the sugar ring is of the pyranoid type and, since the glycosidic link in biosynthetic glucuronides is of the β-type (for literature see Pryde & Williams [1934]), the glucuronide excreted by rabbits after administration of phenolsulphonamide appears to be *p*-sulphonamidophenyl-β-*d*-glucopyranuronoside (I).

Experimental

Phenol-*p*-sulphonamide was easily prepared by diazotization of sulphanilamide followed by heating of the acid diazo solution on a boiling water bath. The sulphonamide was extracted from the solution with ether. The yield was 38 g. from 50 g. sulphanilamide. The M.P. after recrystallization from water was 176° (lit. M.P. 176°).

The ethereal sulphate conjugation

The percentage of phenolsulphonamide excreted as ethereal sulphate was determined exactly as described in previous papers on the sulphate conjugation

of substituted phenols and sulphanilamide [Williams, 1938; Shelswell & Williams, 1940]. The results are given in Table 1.

Table 1.* *The conjugation of phenolsulphonamide with sulphate in the rabbit*

Rabbit no.	Dose mg./kg.	Amount of phenolsulphonamide conjugated with sulphate: %		mg./kg.
77	100	61·1		53
11		59·1	av. 53	
9		47·5		
76		44·9		
9	250	51·5		110
63		42·5	av. 44	
62		38·1		
9	500	38·3		165
76		34·9	av. 33	
70		26·8		
9	750	30·2		203
77		29·7	av. 27	
63		25·5		
76		23·9		

* The figures in this table will include any sulphate conjugated with the catechol derivative.

Table 2

Compound	Dose g./kg.	Sulphate conjugation
1-Hydroxybenzene-4-sulphonic acid	0·5	0
1-Hydroxy-2-aminobenzene-4-sulphonic acid	0·5	0
1-Amino-2-hydroxybenzene-4-sulphonic acid	0·5	0
1-Amino-3-hydroxybenzene-4-sulphonic acid	0·5	0
Toluene-*p*-sulphonamide	0·1	0

The glucuronic acid conjugation

Barium p-*sulphonamidophenylglucuronate.* Rabbits (2–3 kg.) were each given by stomach tube 2 g. of phenolsulphonamide dissolved in 10–20 ml. warm water. The urine was collected during 2 days. It was made faintly acid with glacial acetic acid and then treated with saturated normal lead acetate solution until precipitation was complete. The solution was filtered and, after neutralization by conc. ammonia, the clear yellow filtrate was completely precipitated with saturated basic lead acetate. The basic lead precipitate was collected on a Büchner funnel, thoroughly washed with water, and, before it had time to dry, was made into a thin cream with water. This suspension was gassed with H_2S and the PbS filtered off under suction. The filtrate was concentrated *in vacuo* to a dark syrup which failed to crystallize. The syrup was now dissolved in water and treated with an excess of $BaCO_3$ and the resulting mixture filtered and washed with water. The filtrate and washings (the volume was kept as small as possible) were now poured into a large volume of alcohol, thereby precipitating the required *barium salt.* Next day the salt was filtered, washed with alcohol or acetone and dried in a desiccator. The alcoholic filtrates from this salt were preserved for further investigation (see below). Purification of the salt was accomplished by repeated precipitation from aqueous solution by alcohol, followed by washing with acetone and drying off the acetone *in vacuo*. The barium salt so obtained was a faintly coloured electrostatic powder, soluble in water, giving a pale reddish-brown solution and an intense naphthoresorcinol

reaction. It showed $[\alpha]_D - 50°$ ($c = 1$, in water). (Found: Ba, 16·6; N, 3·1 %. $(C_{12}H_{14}O_9NS)_2Ba$ requires Ba, 16·5; N, 3·3 %.) The yield of the crude salt was 1–1·25 g./g. of phenolsulphonamide fed. Aqueous solutions of the crude salt gave an intense green colour with $FeCl_3$, but the pure compound gave no colour. Removal of the Ba and subsequent suitable treatment gave the free glucuronide as a dark hard resin.

Hydrolysis of the barium salt. The salt (1 g.) was dissolved in 10 ml. water and the barium quantitatively removed with $2N$ H_2SO_4. To the filtrate and washings (40 ml.) from the $BaSO_4$, 10 ml. conc. HCl were added, thus making the solution about 2·5 N with respect to HCl. The solution was now gently boiled under reflux for 3 hr. and, after cooling and filtering to remove some black insoluble material, the strongly reducing solution was exhaustively extracted with ether. Removal of the ether left a sticky crystalline residue. Some of the crystals were separated and recrystallized from water. They were phenolsulphonamide, M.P. 170° (not depressed by authentic material, M.P. 176°) and gave the weak purple colour with $FeCl_3$ characteristic of the sulphonamide. The rest of the residue was dissolved in Na_2CO_3 solution and acetic anhydride added with cooling. After a short while, p-*acetoxybenzenesulphonamide* separated and was recrystallized (needles, M.P. 158°)[1] from 50 % aqueous alcohol. Since this acetyl derivative has not been previously described an authentic specimen was prepared. It formed long needles from 50 % aqueous alcohol, M.P. 159°, not depressed by the compound obtained through hydrolysis of the barium salt. (Found: N, 6·7 %. $C_8H_9O_4NS$ requires N, 6·5 %.) Another sample of the barium salt was hydrolysed and the crystalline residue obtained by ether extraction was benzoylated with benzoyl chloride and NaOH. A gum was obtained which, on crystallization from spirit, gave *p*-benzoyloxybenzenesulphonamide, M.P. 237°, as white plates. It did not depress the M.P. of an authentic sample, M.P. 238°. (Schreinemakers [1897] gives M.P. 234–236°.)

Methylation of the barium salt. The barium salt (5 g.) was dissolved in 50 ml. water and acetone was added until a slight permanent precipitate was obtained. The solution was then methylated at 35° with 25 ml. methyl sulphate and 60 ml. 30 % NaOH added dropwise with stirring. The stirring was continued for 5 hr. with occasional additions of acetone and then at 70° to destroy excess methyl sulphate and remove acetone. The solution was now cooled in ice, neutralized with $5N$ H_2SO_4 and made slightly acid to Congo red. Methylated spirit was added to precipitate Na_2SO_4 which was filtered under suction and thoroughly washed with spirit. The filtrate and washings were made slightly alkaline with dilute NaOH and concentrated to a thin syrup. The latter was now taken up in a small amount of $5N$ H_2SO_4 and the sticky precipitate formed was exhaustively extracted with chloroform. The chloroform solution was washed with water, dried over anhydrous Na_2SO_4 and then concentrated to an acid syrup (2·76 g.) which failed to crystallize. The syrup was now given three treatments with methyl iodide and silver oxide, i.e. until a constant methoxyl value was obtained. A yellow viscous syrup (2·2 g.) was obtained which could not be crystallized. This is presumably the *methyl ester* of 2:3:4-*trimethyl* p-*sulphondimethylamidophenylglucuronide*. It showed $[\alpha]_D^{21°} - 51·3°$ ($c = 1$, in $CHCl_3$). (Found: OMe, 29·4 %. $C_{18}H_{27}O_9NS$ requires OMe, 28·6 %.)

2:3:4-Trimethyl p-*sulphondimethylamidophenylglucuronamide.* The above methyl ester (0·5 g.) was dissolved in 5 ml. dry methyl alcohol and the solution saturated with dry ammonia at 0°. After 20 hr. in a desiccator at room temp. the solvent was evaporated and the residue crystallized on the addition of ether.

[1] Melting points are uncorrected.

The *amide* was recrystallized from acetone-ether. It formed long needles, M.P. 154–5°, slightly soluble in ether, but soluble in warm water, alcohol and acetone (yield 90 % theory). It showed $[\alpha]_D^{21°} -42·3°$ ($c=0·94$ in abs. alcohol) and $[\alpha]_D^{20°} -52·2°$ ($c=1$, in water). (Found: C, 48·6; H, 6·1; N, 7·0; OMe, 23·1. $C_{17}H_{26}O_8N_2S$ requires C, 48·8; H, 6·2; N, 6·7; OMe, 22·25 %.)

Experiments with the oxidation product of phenolsulphonamide

The alcoholic filtrates and washings from the preparation of the barium salt were concentrated *in vacuo* to a syrup. This syrup was dissolved in absolute alcohol, leaving behind a quantity of the barium salt which had escaped precipitation in the original preparation. Three concentrations and solutions in alcohol were necessary to remove all the barium salt; finally, a syrup completely soluble in alcohol was obtained. The amount of this syrup was 1–1·5 g. from six rabbits fed with 2 g. each of phenolsulphonamide. The syrup was soluble in water and its solution did not reduce Fehling's solution, but reduced ammoniacal silver nitrate vigorously on warming. It gave the typical catechol $FeCl_3$ reaction (green, blue-green, purple, red on increasing the alkalinity) and gave an intense naphthoresorcinol reaction for glucuronic acid. One sample was methylated with methyl sulphate and alkali and the product extracted with ether. The ethereal solution was evaporated to a thick liquid and distilled. A brownish distillate was obtained, but no veratrole (dimethyl catechol) could be detected in it and the nature of the distillate was not discovered. Another methylated sample was subjected to treatment with various solvents in an attempt to crystallize out veratrole-4-sulphonamide, an authentic sample [Paul, 1906] of which had been prepared for comparison. No veratrolesulphonamide was obtained. Another sample of the syrup (1·5 g.) was hydrolysed by boiling under reflux with 20 ml. of 2*N* HCl. The hydrolysate was then extracted with ether in a continuous extractor. On evaporation of the extract a brownish crystalline residue was obtained which gave an intense green colour with $FeCl_3$. Treatment of this residue with Na_2CO_3 and acetic anhydride produced *p*-acetoxybenzenesulphonamide. The isolation of this compound, together with the fact that the hydrolysate was very strongly reducing, indicated that the syrup was mainly *p*-sulphonamidophenylglucuronide, containing a small amount of the catechol derivative. The isolation of the latter is now being tackled by other methods in conjunction with other work of a similar nature in this Laboratory.

SUMMARY

The fate of *p*-hydroxybenzenesulphonamide (phenol-*p*-sulphonamide) in the rabbit has been studied. At a dose level of 0·75 g./kg. about 50 % of the dose is excreted conjugated with glucuronic acid, about 30 % with sulphuric acid and a small amount is oxidized to a catechol derivative, probably catecholsulphonamide. No free phenolsulphonamide was excreted.

A study was also made of the ethereal sulphate conjugation of phenolsulphonamide at various dose levels and it was found that the percentage conjugated fell with increasing dose.

The conjugated glucuronide of phenolsulphonamide was isolated as a barium salt and characterized by means of the crystalline amide of the 2:3:4-trimethyl-*p*-sulphondimethylamidophenyl-β-*d*-glucuronide.

It has been shown that phenol-*p*-sulphonic acid, 1-hydroxy-2-amino-, 1-amino-2-hydroxy- and 1-amino-3-hydroxy-benzene-4-sulphonic acids do not conjugate with glucuronic and sulphuric acids in the rabbit and are probably excreted unchanged.

REFERENCES

Baumann & Herter (1877–8). *Hoppe-Seyl. Z.* **1**, 244.
—— & Preusse (1879). *Hoppe-Seyl. Z.* **3**, 156.
Buttle, Gray & Stephenson (1936). *Lancet*, **1**, 1289.
Flaschenträger, Bernhard, Löwenberg & Schläpfer (1934). *Hoppe-Seyl. Z*, **225**, 157.
Friedländer (1896). *Fortschr. Teerfarb.* **3**, 59.
—— (1911). *Fortschr. Teerfarb.* **9**, 148.
Horn (1936). *Hoppe-Seyl. Z.* **238**, 84.
Jaffe & Hilbert (1888). *Hoppe-Seyl. Z.* **12**, 295.
Kleine (1896–7). *Hoppe-Seyl. Z.* **22**, 327.
Paul (1906). *Ber. dtsch. chem. Ges.* **39**, 2773.
Pryde & Williams (1933). *Biochem. J.* **27**, 1197.
—— —— (1934). *Biochem. J.* **28**, 133.
Schreinemakers (1897). *Rec. Trav. chim. Pays-Bas*, **16**, 423.
Scudi (1940). *Science*, **91**, 486.
Shelswell & Williams (1940). *Biochem. J.* **34**, 528.
Thorpe & Williams (1940). *Nature, Lond.*, **146**, 686.
—— —— (1941). *Biochem. J.* **35**, 61.
—— —— & Shelswell (1941). *Biochem. J.* **35**, 52.
Wiley (1938). *J. biol. Chem.* **124**, 627.
Williams (1938). *Biochem. J.* **32**, 878.

Note added 27 May 1941. The catechol derivative mentioned above has now been identified as catechol-4-sulphonamide, since suitable treatment, including methylation, of ether extracts of the hydrolysed urine gave crystalline *veratrole-4-sulphondimethylamide*, M.P. 105°, identical with a synthetic sample. Synthetic *catechol-4-sulphonamide* has also been prepared and found to be a non-crystalline resin, although *catechol-4-sulphonanilide* is crystalline, M.P. 221°. A description of this work will be reported when completed.