excess tungstic acid in the strongly acid solution in which the oxidation and bromination of citric acid is carried out. This does not interfere with the reaction, but it makes the removal of the aqueous layer by blowing out impossible. Instead, the petroleum layer is sucked off.

Cerebrospinal fluid. The c.s.f. (1 vol.) is mixed with 30 % trichloroacetic acid (0·1 vol.), and 1–3 ml. of the filtrate are directly analysed without further pretreatment. Range: 40–80 μg./ml. (cf. Benni, Larsson & Thunberg, 1943).

Urine. Protein, if present, is removed by precipitation with trichloroacetic acid, turbidities by filtration, acetoacetic acid by boiling with dilute acid, and aromatic compounds, such as salicylates, by preliminary bromination. No preliminary treatment whatever is required for normal urines which are analysed directly after suitable dilution. Samples of 1–5 ml. of urine diluted fivefold usually give satisfactory readings. Recovery of added citric acid was quantitative.

SUMMARY

1. A modification of the colorimetric estimation of citric acid is described in which permanganate is replaced by vanadic acid. This gives increased simplicity and speed of operation, and greater freedom from interference by other oxidizable substrates. The yield of pentabromoacetone is almost quantitative.

2. Some recent modifications of the colorimetric estimation of pentabromoacetone are critically examined.

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Studies in Detoxication

27. THE ORIENTATION OF CONJUGATION IN THE METABOLITES OF 4-CHLOROCATECHOL AND 4-CHLORORESORCINOL, WITH SOME OBSERVATIONS ON THE FATE OF (+)-ADRENALINE, PROTOCATECHUIC ACID AND PROTOCATECHUIC ALDEHYDE IN THE RABBIT

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(*Received* 6 *May* 1949)

Garton & Williams (1948) have shown that catechol gives rise to monoconjugates, *o*-hydroxyphenylglucuronide and *o*-hydroxyphenylsulphuric acid, in the rabbit. Quinol and resorcinol also form monoconjugates in the rabbit (Garton & Williams, 1949). The object of the present work was to find out which of the two hydroxyls becomes conjugated if a third group X is present in a position *para* to one of the hydroxyls in catechol compounds. We first studied protocatechuic aldehyde ($X = CHO$), protocatechuic acid ($X = COOH$) and (+)-adrenaline

$$(X = CH(OH).CH_2NHMe).$$

These three compounds formed glucuronides in the rabbit, but we did not succeed in isolating them in a crystalline state. The structure of these glucuronides could not therefore be rigidly proved. With 4-chlorocatechol ($X = Cl$), however, we succeeded in isolating in high yield a crystalline monoglucuronide whose structure could be determined. The structural

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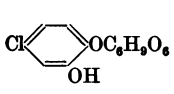
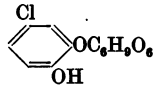
problem amounted to proving that the isolated glucuronide was either 4-chloro- (I) or 5-chloro-2-hydroxyphenylglucuronide (II):



(I)



(II)

Since chlorocatechol formed a monoconjugate, it was interesting also to know whether 4-chlororesorcinol and chloroquinol formed monoconjugates, because the formation of monoconjugates in these compounds raises interesting questions concerning the orientation of conjugation. The study of the isolation and structure of 4-chlororesorcinol glucuronide is also included in this paper. In this case the structural problem involved consisted in proving that 4-chlororesorcinol glucuronide was either 4-chloro- or 6-chloro-3-hydroxyphenylglucuronide. Our studies on chloroquinol will be reported in a future paper.

METHODS AND MATERIALS

Materials. 4-Chlorocatechol hemihydrate (m.p. 81°) was prepared according to Willstätter & Müller (1911), and 4-chlororesorcinol (m.p. 105°) according to Reinhard (1878) (cf. Moore, Day & Suter, 1934). (+)-Adrenaline (m.p. 210°; $[\alpha]_D^{20°}$ +50° in 0·1 N-HCl) was the gift of Burroughs Wellcome and Co. Protocatechuic aldehyde (m.p. 154°) was a commercial sample. Protocatechuic acid (m.p. 197–198°) was prepared in good yield by adding vanillin in small quantities to fused KOH, the method being a slight modification of that of Tiemann & Haarmann (1874).

Animals. The rabbits used were kept on a diet of either 50 g. or 75 g./day of Lever's cubes with water *ad lib.*

Analytical methods. Ethereal sulphates and glucuronic acid in urine were determined as in earlier papers in this series (Williams, 1938; Hanson, Mills & Williams, 1944).

Reference compounds. 4-Chloro-2-methoxyphenyl benzoate (m.p. 78–79°) was prepared according to Jona & Pozzi (1911).

Synthesis of 4-chloro-3-methoxyphenol. This compound was originally described by von Auwers & Pohl (1914–15), who obtained it in small yield by a lengthy procedure. We prepared it by a Sandmeyer reaction from 4-amino-3-methoxyphenol. Resorcinol monomethyl ether (Dey, 1935) was converted through *p*-sulphophenylazo-*m*-methoxyphenol to 4-amino-3-methoxyphenol (Heidelberger & Jacobs, 1919). CuCl obtained from 12·5 g. $CuSO_4.5H_2O$ was dissolved in 20 ml. conc. HCl. This solution was added to the solution of the diazonium compound obtained by treating 3 g. 4-amino-3-methoxyphenol dissolved in 30 ml. 5–6 N-HCl with 1·5 g. $NaNO_2$ at −5°. The mixture was then refluxed for 45 min., whereby N_2 was liberated and a dark-coloured oil separated. This oil was extracted with ether and the extract well washed with water. The phenol was now transferred to 2 N-NaOH by extraction and finally the alkaline extract was acidified with 2 N-HCl and the phenol extracted with ether. After drying with anhydrous Na_2SO_4, treating with charcoal and evaporating the ether, a dark-coloured gum was obtained which slowly crystallized (yield, 1·4 g.). On distillation in a vacuum (140–150°/<1 mm.) 0·8 g. of 4-chloro-3-methoxyphenol was obtained. After recrystallization from acetone-water the phenol was obtained as white plates, m.p. 77–78°. (Found: OMe, 19·6. Calc. for $C_7H_7O_2Cl$: OMe, 19·6%.) Von Auwers & Pohl (1914–15) give m.p. 79–80°.

4-Chloro-3-methoxyphenyl p-toluenesulphonate was obtained in good yield in the usual manner using aqueous NaOH and *p*-toluenesulphonyl chloride. It formed plates, m.p. 65–66°, after recrystallization from aqueous ethanol. (Found: C, 53·9; H, 4·2; Cl, 11·0; S, 10·0. $C_{14}H_{13}O_4SCl$ requires C, 53·8; H, 4·2; Cl, 11·3; S, 10·3%.)

RESULTS

Table 1 shows that both 4-chlorocatechol and 4-chlororesorcinol were excreted completely conjugated with glucuronic and sulphuric acids, the ratio glucuronide/ethereal sulphate being about 6 for chlorocatechol and 3 for chlororesorcinol. With protocatechuic aldehyde and acid, however, there was by no means a complete conjugation, for just over 40 % of the aldehyde and about 30 % of the acid could be accounted for by conjugation.

In the case of the aldehyde, large amounts of free protocatechuic acid could be isolated from the acidified urine by extraction with ether. This acid no doubt accounts for the rest of the aldehyde. It was also found that two glucuronides were being excreted, one which could be isolated as a gum and appeared to be a protocatechuic acid glucuronide and the other a protocatechuic aldehyde glucuronide which could be isolated as a 2:4-dinitrophenylhydrazone. As these glucuronides were difficult to purify, they were not further studied.

In the case of protocatechuic acid, a considerable amount of this acid can be extracted from the acidified urine with ether. In one experiment we recovered in crystalline form some 30 % of the dose. It appears, therefore, that about one-third of the acid fed appears in the urine conjugated and the rest in the free state.

The results with (+)-adrenaline are different from all the others. The ethereal sulphate figures are probably not significant, whereas the glucuronic acid figures suggest that some 20 % of the adrenaline fed was excreted as glucuronides. We spent a considerable time studying the nature of these glucuronides, but we did not reach definite conclusions because the glucuronides were non-crystalline. We were, however, able to show that two glucuronides were being excreted, one being a (+)-adrenaline glucuronide giving (+)-adrenaline on hydrolysis, and the other a nitrogen-free glucuronide containing a catechol-like substance, as indicated by absorption spectrophotometry. The second glucuronide no doubt arises from deamination products of adrenaline and it can be separated chromatographically from the first glucuronide. Colour tests indicated that, in the

Table 1. *Glucuronic acid and ethereal sulphate excretion of rabbits receiving 4-substituted catechols orally*

Compound fed	Rabbit no.	Wt. (kg.)	Dose (mg.)	Ethereal sulphate (mg. SO_3/day) Average normal	Ethereal sulphate (mg. SO_3/day) 'Extra'	Glucuronic acid (mg./day) Average normal	Glucuronic acid (mg./day) 'Extra'	Dose (%) excreted as Ethereal sulphate	Dose (%) excreted as Glucuronide
4-Chlorocatechol*	143	2·9	430	35·8	31·2	247	493	14·0	91·0
	145	2·5	370	34·8	41·2	268	407	21·4	87·0
	146	2·15	320	30·0	18·0	217	353	10·5	84·5
4-Chlororesorcinol†	128	3·0	450	18·2	51·1	124	476	20·5	78·8
	129	3·0	450	19·5	71·4	151	459	28·6	76·0
	130	3·0	450	18·3	57·4	182	484	23·0	80·0
Protocatechuic aldehyde†‡	83	3·1	775	18·8	90·7	121	273	20·2	25·1
	114	2·7	636	20·7	50·8	108	242	14·1	27·1
	117	2·4	601	20·2	51·3	123	239	14·7	28·1
Protocatechuic acid†‡	80	3·1	774	25·3	62·1	—	—	15·5	—
	81	2·45	613	24·0	44·2	—	—	13·9	—
	124	3·3	825	29·3	40·1	80	160	9·4	15·4
	125	3·6	900	11·6	76·8	88	219	16·4	19·4
	126	3·6	900	17·3	53·1	102	220	11·4	19·4
(+)-Adrenaline†	68	2·85	710	17·0	13·3	147	142	4·3	18·9
	102	2·9	730	22·6	21·5	148	153	6·7	19·7
	101	2·85	713	33·0	0·0	—	—	0·0	—
	60	2·55	510	25·5	0·0	160	103	0·0	19·1
	67	2·5	500	30·8	15·0	149	157	6·9	29·6
	68	2·9	580	—	—	174	126	—	20·5

* Diet: 75 g. Lever's cubes/day.
† Diet: 50 g. Lever's cubes/day.
‡ The results quoted for these compounds were obtained by Dr G. A. Garton.

gummy (+)-adrenaline glucuronide, the glucuronic acid molecule is attached to one of the phenolic hydroxyl groups of adrenaline.

The metabolites of 4-chlorocatechol

The structure of 4-chlorocatechol glucuronide

The glucuronide gum. Twelve rabbits received collectively 9 g. of chlorocatechol hemihydrate (0·75 g. each) dissolved in water. The 24-hr. urine (1200 ml.) appeared normal in colour and gave an intense Tollens test for glucuronic acid but no reaction with $FeCl_3$ or Benedict reagent. It was made acid to congo red with dilute H_2SO_4 and saturated with $(NH_4)_2SO_4$. To the mixture 50 ml. of 1 : 5 ethanol : ether were added, and the precipitated salts filtered off. The clear filtrate was now exhaustively extracted in a separating funnel with 200 ml. portions of the 1 : 5 ethanol : ether mixture. After drying over anhydrous Na_2SO_4, the yellowish extract was treated with charcoal and filtered. The filtrate was taken to a dark gum (15 g.) *in vacuo* at a low temperature. This gum, which is presumably *4-chlorocatechol glucuronide*, did not crystallize. It formed a crystalline *salt* (m.p. 108–109°) with benzylamine which appeared to contain two molecules of benzylamine. This salt was, however, unstable. 4-Chlorocatechol itself formed a crystalline salt (m.p. 61–62°) with benzylamine. These salts were not further investigated owing to their instability.

The glucuronide gum gave an intense Tollens test but no colour with $FeCl_3$. With 2:6-dichloroquinonechloroimide it gave a deep blue colour at pH 7. The latter test indicates the presence in the compound of a free phenolic hydroxyl group with the position *para* to it unsubstituted.

4-Chloro-2-methoxyphenylglucuronamide. The above gum (12 g.) was dissolved in 100 ml. absolute ethanol and repeatedly methylated with excess ethereal diazomethane for 12 hr. periods at room temperature until the colour test with 2:6-dichloroquinonechloroimide was negative. Filtration and removal of the solvents left a clear gum which did not crystallize. The gum appears to be *4-chloro-2-methoxyphenylglucuronide methyl ester.* (Found: OMe, 19·4. $C_{14}H_{17}O_8Cl$ requires OMe, 17·8%.)

This ester (12 g.) was dissolved in the minimum of absolute methanol and the solution saturated with dry NH_3 at 0°. The flask was stoppered and kept at 0° for 12 hr. After that time the whole had set to a gelatinous mass, which crystallized on adding a few drops of acetone and scratching. The crystals (9·2 g.; m.p. 216°) were filtered and washed with 30% ethanol from which solvent it crystallized as long colourless narrow plates, m.p. 218° and $[\alpha]_D^{20°} - 96·15°$ ($c = 0·2$ in 50% aqueous acetone). The *4-chloro-2-methoxyphenyl-β-D-glucuronamide monohydrate* was insoluble in cold water, ether, light petroleum and benzene, slightly soluble in ethanol, soluble in acetone-water mixtures and readily soluble in hot ethanol, water and acetone. (Found: C, 45·0; H, 5·2; N, 3·9; Cl, 10·0; H_2O, 4·6. $C_{13}H_{16}O_7NCl.H_2O$ requires C, 44·4; H, 5·2; N, 4·0; Cl, 10·1; H_2O, 5·1%.) The yield of this amide corresponded to 60% of the chlorocatechol fed.

Hydrolysis of the above amide and isolation of 4-chloroguaiacol. The amide (1·5 g.) was heated on a boiling water bath for 5 hr. with 50 ml. N-HCl. The amide gradually dissolved and eventually a brown oil separated. The mixture was cooled and extracted with ether. After keeping overnight over anhydrous Na_2SO_4, the ethereal extract was treated with charcoal and filtered. The now pale-yellow filtrate was evaporated at room temperature to a partly crystalline solid

consisting of chloroguaiacol. This was benzoylated with benzoyl chloride and NaOH. On pouring the benzoylated mixture into water a solid (0·9 g.) separated. This was recrystallized (charcoal) from ethanol, yielding 0·65 g. of colourless plates, m.p. 72°. Further recrystallization from ethanol raised the m.p. to 78–79° and the compound was identified as 4-chloro-2-methoxyphenyl benzoate. (Found: OMe, 11·9. Calc. for $C_{14}H_{11}O_3Cl$: OMe, 11·8%.) It did not depress the m.p. of authentic 4-chloro-2-methoxyphenyl benzoate (m.p. 78–79°) prepared according to Jona & Pozzi (1911). The other isomer, 5-chloro-2-methoxyphenyl benzoate, melts at 56–58° (Jona & Pozzi, 1911).

The ethereal sulphate of chlorocatechol

The urine of ten rabbits which had received collectively 10 g. of chlorocatechol was collected for 2 days (2·5 l.) and then concentrated to 500 ml. under reduced pressure. The concentrate was saturated with $(NH_4)_2SO_4$ and extracted with 4 × 300 ml. acetone. These extracts, after the addition of 2 g. K_2CO_3 to maintain the solution alkaline, were reduced to 200 ml. *in vacuo*. At this stage the material contained ethereal sulphate and some glucuronide, but gave no test with $FeCl_3$. Much of the glucuronide was thrown out as a gum on pouring the extract into 1 l. dry acetone. The acetone solution was now reduced to 100 ml. and again poured into 1 l. dry acetone. In this way the whole of the glucuronide was removed and the final acetone solution was now reduced to 100 ml. This solution was now diluted with 300 ml. absolute ethanol and then treated with an ethanolic solution of anhydrous oxalic acid. The precipitated urea oxalate was removed, the filtrate made alkaline with ethanolic KOH and then reduced to small bulk, and the whole process repeated. In this way the urea present was removed. Excess oxalic acid was removed by making the concentrate alkaline with ethanolic KOH and filtering off the precipitated potassium oxalate. Finally, a dark brown syrup was obtained which reacted strongly for ethereal sulphates, but gave negative tests for glucuronide and inorganic sulphate. The $FeCl_3$ test was negative, but became intensely positive for catechol on acid hydrolysis. With 2:6-dichloroquinonechloroimide the gum gave an intense blue colour which suggested that the ethereal sulphate was 4-chloro-2-hydroxyphenylsulphuric acid. We were unable to induce the gum to crystallize.

The gum was therefore methylated in the usual way with methyl sulphate and NaOH until the blue colour given by 2:6-dichloroquinonechloroimide had disappeared, and in this way 1·5 g. of a methylated gum containing ethereal sulphate was obtained. The gum was hydrolysed by boiling for 20 min. with 3N-H_2SO_4 and the phenol set free was extracted with ether. The ether yielded 0·8 g. of a reddish gum (Found: OMe, 16·0. Calc. for $C_7H_7O_2Cl$: OMe, 19·6%), which is presumably crude chloromethoxyphenol. This material was benzoylated, but the product would not crystallize. The benzoylated gum was therefore distilled at 80° in a vacuum (<1 mm.) and the straw-coloured distillate crystallized on seeding with authentic 2-methoxy-4-chlorophenyl benzoate. On recrystallization from ethanol 14 mg. of 2-methoxy-4-chlorophenyl benzoate m.p. and mixed m.p. 79° were obtained. (Found: OMe, 11·6. Calc. for $C_{14}H_{11}O_3Cl$: OMe, 11·8%.) This experiment suggests that the ethereal sulphate in chlorocatechol urine is 4-chloro-2-hydroxyphenylsulphuric acid.

THE METABOLITES OF 4-CHLORORESORCINOL

4-chlororesorcinol glucuronide

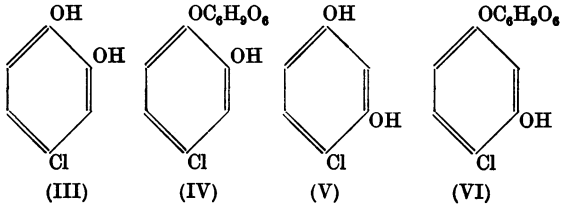
The glucuronide gum. A total of 5·4 g. of 4-chlororesorcinol was fed to six rabbits. The urine (850 ml.) was non-reducing, gave no colour with $FeCl_3$, but gave a strong naphthoresorcinol test. The glucuronide gum (9 g.), presumably *4-chlororesorcinol glucuronide*, was obtained by ether ethanol extraction as described for chlorocatechol glucuronide.

4-Chloro-3-methoxyphenylglucuronamide. Repeated methylation of the gum with ethereal diazomethane yielded the non-crystalline ester, *4-chloro-3-methoxyphenylglucuronic acid methyl ester*. (Found: OMe, 17·9. $C_{14}H_{17}O_8Cl$ requires OMe, 17·8%.) Treatment of the ester in dry methanol with gaseous NH_3 yielded 6·2 g. (equivalent to 50% of the dose) of crude *4-chloro-3-methoxyphenyl-β-D-glucuronamide monohydrate*. The amide formed small plates from water with m.p. 214–215° and $[\alpha]_D^{19°} = -103·3°$ ($c = 0·2$ in 50% aqueous acetone). (Found: C, 44·7; H, 5·3; N, 4·1; OMe, 9·0; H_2O, 4·7. $C_{13}H_{16}O_7NCl.H_2O$ requires C, 44·4; H, 5·2; N, 4·0; OMe, 8·8; H_2O, 5·1%.)

Hydrolysis of the above amide and isolation of 4-chloro-3-methoxyphenol. The above glucuronamide (1·4 g.) was hydrolysed by heating on the water bath for 6 hr. with 50 ml. 2N-HCl. The hydrolysate was black in colour and a black gum separated. It appears that the hydrolysis is complicated by the combination of glucuronic acid and/or its degradation products with the phenol set free, as was found in the case of resorcinol glucuronide (Garton & Williams, 1949). The whole of the hydrolysate was exhaustively extracted with ether. The extracts were washed with water and extracted with 2N-NaOH. The alkaline extract was now acidified with 2N-HCl and the phenol extracted with ether. After drying with anhydrous Na_2SO_4 and treating with charcoal, the ether was evaporated leaving 0·55 g. of a black gum. This gum was now subjected to vacuum distillation (<1 mm.). The fraction distilling at 150–160° (bath temperature) was a pale straw-coloured viscous oil (0·18 g.) which crystallized. On recrystallization from acetone-water, the material was identified as 4-chloro-3-methoxyphenol, m.p. and mixed m.p. 77–78°. (Found: OMe, 19·3. Calc. for $C_7H_7O_2Cl$: OMe, 19·6%.) The *p*-toluenesulphonate was prepared and on recrystallization from aqueous ethanol yielded plates m.p. and mixed m.p. 65–66° with the authentic sample prepared above.

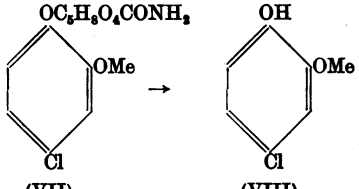
DISCUSSION

It is clear that the glucuronides excreted by rabbits receiving 4-chlorocatechol (III) and 4-chlororesorcinol (V) are monoglucuronides, and can be described as 4-chloro-2-hydroxyphenylglucuronide (IV) and 4-chloro-3-hydroxyphenylglucuronide (VI), respectively.

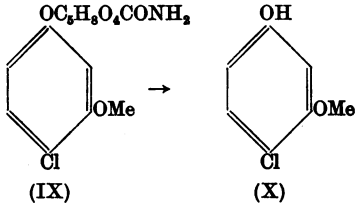


(III) (IV) (V) (VI)

Neither of these compounds was obtained in a crystalline state, but they were readily characterized as the crystalline amides, 4-chloro-2-methoxyphenyl-β-D-glucuronamide (VII; monohydrate, m.p. 218°) and 4-chloro-3-methoxy-β-D-glucuronamide (IX; monohydrate, m.p. 214–215°) which were obtained in excellent yields. The position of conjugation was determined by the hydrolysis of these amides and identification of the resulting chloromethoxyphenols. The amide (VII) yielded 4-chloro-2-methoxyphenol (VIII) characterized as the benzoate, and (IX) yielded 4-chloro-3-methoxyphenol (X) characterized as the *p*-toluenesulphonate.



(VII) (VIII)



(IX) (X)

The present results raise a number of interesting questions concerning the orientation of glucuronic acid conjugation. It has already been shown that in the rabbit catechol, resorcinol and quinol are conjugated on one hydroxyl only (Garton & Williams, 1948, 1949). This has now been shown to apply in the cases of 4-chlorocatechol and 4-chlororesorcinol, but with these compounds a further point arises because the two hydroxyls, in each case, are differently orientated in relation to the third substituent, chlorine. In both cases conjugation appears on the hydroxyl which is *para* to the chloro group. In 4-chlorocatechol the hydroxyl group *meta* to the chloro group is unconjugated, whereas in 4-chlororesorcinol the unconjugated hydroxyl group is *ortho* to the chloro group. It is thus clear that conjugation takes place at the hydroxyl which is farthest from the chloro group. Before, however, we can make definite statements about this orientation we must await results with chloroquinol in which one hydroxyl is *ortho* and the other *meta* to the chloro group. If the orientation of conjugation is simply a matter of distance then it can be predicted that chloroquinol will be conjugated at the *meta* hydroxyl.

It is interesting to compare the conjugation of chlorocatechol and chlororesorcinol with that of catechol and resorcinol. The relevant figures are given in Table 2 which shows that the total conjugation of the chloro compounds is higher than the dihydroxybenzenes. This is clearly the case with the resorcinols, although the ratio glucuronide/ethereal sulphate for chlororesorcinol is approximately the same as for resorcinol. In the case of resorcinol, Garton & Williams (1949) found that at least 10% of the resorcinol fed could be recovered in the urine in the free state. With the two catechols, there appears to be a slight suppression of sulphate conjugation and a slight increase in glucuronic acid conjugation, on going from catechol to 4-chlorocatechol. However, in view of the variations in the individual results it would be safer to conclude that the introduction of the chloro group into the 4-position of catechol has but little influence on the conjugation of catechol, whereas the introduction of the chloro group into the 4-position of resorcinol increases both the ethereal sulphate and the glucuronic acid conjugations.

SUMMARY

1. A study has been made of the conjugation of 4-chlorocatechol and 4-chlororesorcinol in the rabbit.

2. About 87% of 4-chlorocatechol is excreted as a glucuronide, which was characterized as the crystalline 4-chloro-2-methoxyphenylglucuronamide and proved to be 4-chloro-2-hydroxyphenylglucuronide. Some 15% of chlorocatechol forms a mono-ethereal

Table 2. *The conjugation of catechol and resorcinol and their 4-chloro derivatives in the rabbit*

Compound	Dose (mg./kg. body wt.)	Dose (%) excreted as: Ethereal sulphate (*E*)	Glucuronide (*G*)	*G*/*E*	Total conjugation (*G*+*E*)
Catechol*	100†	18	70	3·9	88
4-Chlorocatechol	150†	15·3	87	5·7	103
Resorcinol‡	100†	13·5	52	3·9	65
4-Chlororesorcinol	150†	24	78	3·3	102

* Figures quoted from Garton & Williams (1948).
† In approximately molecular proportions.
‡ Figures quoted from Garton & Williams (1949).

sulphate which is 4-chloro-2-hydroxyphenylsulphuric acid.

3. Similarly, 4-chlororesorcinol forms 78% of 4-chloro-3-hydroxyphenylglucuronide, characterized as 4-chloro-3-methoxyphenylglucuronamide. Some 24% is excreted as ethereal sulphate.

4. The orientation of conjugation in these two compounds is discussed, and it appears that conjugation takes place at the hydroxyl group *para* to the chloro group.

5. (+)-Adrenaline forms 20% of glucuronide but practically no ethereal sulphate. The results suggest that (+)-adrenaline is conjugated on one of its phenolic hydroxyl groups.

6. With protocatechuic acid, some 30% is conjugated and the rest is excreted unchanged.

7. Protocatechuic aldehyde forms two glucuronides, is more highly conjugated than the acid and is largely transferred to protocatechuic acid and its conjugates.

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Occurrence of Cytochrome and Coproporphyrin in Mycobacteria

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Yaoi & Tamiya (1928) and Fujita & Kodama (1934) observed a four-banded spectrum in tubercle bacilli, the bands corresponding closely to those of reduced cytochrome in yeast or heart muscle. Frei, Riedmüller & Almasy (1934) confirmed the description in the case of *Mycobacterium tuberculosis hominis*, but observed a three-banded spectrum lacking component c in strains of *Mycobact. avium* and *Mycobact. tuberculosis bovis*. In this laboratory it has been noticed that the absorption bands visible in suspensions of acid-fast saprophytes vary in different species, some of which exhibit a distinct narrow band at 624 mμ. This band could be attributed to the presence of cytochrome a_2; alternatively, it could be due to free porphyrin, the other bands of which are obscured by the cytochrome spectrum.

Fischer & Fink (1925) extracted from heat-killed tubercle bacilli a pigment that was identified spectroscopically as a metal complex of coproporphyrin (bands centred at 522·6 and 560·4 mμ.). The spectrum of free coproporphyrin was not visible. The same authors, working with extracts of crude tuberculin, observed intense absorption bands attributed to protoporphyrin but 'only a very weak coproporphyrin spectrum'. Since the culture media were not known to be free from porphyrin initially, Fischer & Fink (1925) did not regard their results as conclusive evidence of porphyrin formation by tubercle bacilli. The occurrence of porphyrin in acid-fast bacteria was demonstrated unequivocally by Dhéré, Glücksmann & Rapetti (1933), who observed the fluorescence spectrum of coproporphyrin in cells of *Mycobact. smegmatis*, *Mycobact. ranae*, *Mycobact. tuberculosis hominis* and *Mycobact. tuberculosis bovis* (strain B.C.G.).

The work described in this paper, originally undertaken with the object of elucidating the atypical spectra of certain mycobacteria, led to the isolation of coproporphyrin III.

METHODS

Organisms. The following were used: *Mycobact. smegmatis* (no. 523), *Mycobact. phlei* (no. 525), *Mycobact.* sp. *Karlinski* (no. 2071), *Mycobact. ranae* (no. 2891) and *Mycobact. stercoris* (no. 3820). The numbers quoted are the catalogue numbers of the National Collection of Type Cultures, Lister Institute, London. The organisms were cultivated at 38° on meat infusion broth containing 2% (w/v) peptone and 5% (v/v) glycerol. Cultures were harvested on the seventh or eighth day, and washed thrice with distilled water (by centrifuga-