steroid. Koepf, Horn, Gemmill & Thorn (1941) observed similarly (using liver slices from normal and adrenalectomized rats and rats adrenalectomized and injected with adrenal cortical hormone) that there was no parallelism between oxygen uptake and power to synthesize carbohydrate. Bartlett, Wick & MacKay (1949) also found with diaphragm that glycogen formation could be increased by insulin or decreased by deoxycorticosterone without affecting oxygen uptake.

From experiments on the whole animal, DOC has usually been associated with influence on mineral metabolism while the steroids with oxygen at C(11) are associated with control over carbohydrate metabolism. In these experiments in vitro, however, we found DOC to be just as potent as 11-dehydrocorticosterone in increasing carbohydrate formation. It has to be remembered that even in vivo there is no hard and fast distinction between the steroids controlling mineral metabolism and those controlling carbohydrate metabolism: thus Harrison & Harrison (1939) showed that daily injection of 1-25 mg. deoxycorticosterone was enough to maintain normal potassium:sodium balance in the serum of rats, while injection of 2-5 mg. maintained normal blood sugar. Young (1944) has pointed out that in experiments in vivo where water-insoluble DOC compounds are used, rate of absorption may play a part.

REFERENCES


SUMMARY

1. The effect of the three pure adrenal cortical steroids, deoxycorticosterone, 11-dehydrocorticosterone and 17-hydroxy-11-dehydrocorticosterone, when added in vitro, upon carbohydrate synthesis in liver slices was investigated.

2. In all cases, whether glycogen or total carbohydrate content was estimated, there was greater increase in carbohydrate content or less carbohydrate disappearance in presence of the steroid than in the controls without added steroid.

3. The presence of the steroids had little or no effect on oxygen uptake.

4. In preliminary experiments, slightly increased concentration of non-protein nitrogen was found in presence of eschatin or of the pure steroids.

I wish to express my hearty gratitude to Dr D. M. Needham, F.R.S., for her untiring advice and continuous help throughout the course of this work.

The pure steroids used were gifts to Dr D. M. Needham and myself: the DOC acetate from Messrs Organon and from Dr Tissières; the DOC succinate from Dr Wettstein of Messrs Ciba; the 11-dehydrocorticosterone from Messrs Organon and the 17-hydroxy-11-dehydrocorticosterone from Dr T. Reichstein. For all these we express our thanks.

I am greatly indebted to Prof. A. C. Chibnall, F.R.S., for the hospitality of his laboratory and to the British Council for a 3-year grant.

Studies in Detoxication

29. THE ORIENTATION OF GLUCURONIC ACID CONJUGATION IN CHLOROQUINOL

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Dodgson & Williams (1949) have shown that 4-chlorocatechol and 4-chloresorescinol form, in rabbits, monoglucuronides (I and II) in which the glucuronic acid residue is attached to the hydroxy group farthest away from the chlorine atom. On these grounds it was predicted that chloroquinol would give rise in the rabbit to the monoglucuronide (III), in which the glucuronic acid is attached to the hydroxy group meta to the chloro group.

In this paper we shall prove that the glucuronide of chloroquinol synthesized in the rabbit has, in fact, structure (III), i.e. that of 3-chloro-4-hydroxy-
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phenylglycuronide. In order to prove this structure and eliminate the alternative structure, 2-chloro-4-hydroxyphenylglycuronide, it was necessary to synthesize a number of reference compounds, particularly the 2- and 3-chloro-4-methoxyphenols and their derivatives.

**EXPERIMENTAL**

3-Chloro-4-methoxyphenol and its derivatives

The synthesis of 3-chloro-4-methoxyphenol was carried out in two ways. (a) 3,4-Dichloronitrobenzene was converted via 3-chloro-4-methoxynitrobenzene to 3-chloro-4-methoxyaniline according to McMaster & Magill (1928). The amine (3 g.) was diazotized at 0° in the usual manner, and the diazonium solution refluxed for 45 min. 3-Chloro-4-methoxyphenol separated as a brown oil which was extracted with ether. The oil was partly purified by extracting the phenol from the ether with 2 N-NaOH, acidifying and then re-extracting with ether. The final ether extract was dried with anhydrous Na2SO4, the ether removed and the residual oil distilled at 6 mm. (bath temp. 160–160°). The solution obtained was obtained as slightly coloured oil which on benzoylation gave the corresponding benzoxide, m.p. 109–110°, described below. (Found: C, 53-9; H, 4-2; Cl, 13-4. C14H11O2Cl requires C, 64-0; H, 4-2; Cl, 13-5%.)

(b) 3-Chloro-4-methoxyphenol was also prepared as described by Irvine & Smith (1927) for the corresponding 3-bromo derivative. 4-Methoxyphenyl benzoxide (3 g.) was dissolved in 25 ml. anhydrous formic acid and poured into a cold solution of 1 g. chloroform in 50 ml. formic acid. The solution lost its colour immediately and a white crystalline precipitate of 3-chloro-4-methoxyphenyl benzoxide separated. After keeping at 0°, the crystals (2 g., 81% of theory) were filtered off. On recrystallization from ethanol-ligroin, the compound was obtained as colourless needles, m.p. 109–110°, soluble in ether and ethanol, but sparingly soluble in light petroleum. (Found: C, 64-1; H, 4-2; Cl, 13-6. C14H11O2Cl requires C, 64-0; H, 4-2; Cl, 13-5%.)

The above benzoxide (1-5 g.) was refluxed with 15 ml. 10% NaOH until it dissolved. The solution was cooled, acidified with 2 N-HCl and extracted with ether (3 x 15 ml.). The ether extract was neutralized with saturated NaHCO3 solution, dried over anhydrous Na2SO4, evaporated and the residue distilled. The phenol distilled at 273°/766 mm. and the distillate solidified on standing (m.p. 42°). (Found: OMe, 19-3. C14H11O2Cl requires OMe, 19-5%). It was sparingly soluble in water and light petroleum but easily soluble in other organic solvents. With FeCl3 it gave a blue colour immediately turning to a grey precipitate.

The phenol (0-2 g.) in 8 ml. 2 N-NaOH was treated with 0-4 g. p-toluene sulphonamide chloride in 8 ml. acetone. After shaking for 30 min. the mixture was poured into 150 ml. water. The crystalline precipitate was recrystallized from ethanol and 3-chloro-4-methoxyphenyl p-toluene sulphonate was obtained as colourless hexagonal plates, m.p. 95–96°. (Found: C, 53-75; H, 4-2; S, 10-0. C16H14O3ClS requires C, 53-85; H, 4-2; S, 10-25%).

2-Chloro-4-methoxyphenyl and its derivatives

In the chlorination of 4-methoxyphenyl benzoate, the chlorine is directed to the 3-position, the methoxyl group being more strongly O-p-directing than the benzoxyl group. However, when 4-methoxyphenol itself is chlorinated, the OH group is the dominant directing group and the chlorine now enters the 2-position (cf. Irvine & Smith, 1927) thus:

![Diagram of 2-Chloro-4-methoxyphenyl and its derivatives](image)

Irvine & Smith (1927) prepared 2-bromo-4-methoxyphenol by the action of bromine on quinol monomethyl ether in organic solvents. Using the same procedure with chlorine in methylene dichloride we found that a mixture of products was produced, but with sulphuryl chloride in ether the expected chlorophenol was obtained.

4-Methoxyphenol (24-6 g.) was refluxed for 0-5 hr. with 18 ml. sulphuryl chloride in 200 ml. ether. The mixture was then washed with water and saturated NaHCO3 solution to remove acid, dried over anhydrous Na2SO4 and the ether distilled. The residual phenol was then distilled at (85–95°/ 0-2–0-3 mm.) to give an oil which solidified on cooling (yield, 24-5 g., 76% of theory). On recrystallization from light petroleum the 2-chloro-4-methoxyphenol formed clumps of white needles, m.p. 42° (depressed on admixture with 3-chloro-4-methoxyphenol, m.p. 42°), sparingly soluble in water and light petroleum but soluble in most organic solvents. (Found: C, 53-1; H, 4-5; Cl, 22-2. C14H11O2Cl requires C, 53-0; H, 4-45; Cl, 22-4%). It gives a blue colour with FeCl3, rapidly fading and forming a grey-brown precipitate. Its benzoxide prepared with 2 N-NaOH and benzoyl chloride, formed needles, m.p. 71°, from ethanol/ligroin. (Found: C, 63-9; H, 4-5; Cl, 13-8. C14H11O2Cl requires C, 64-0; H, 4-2; Cl, 13-5%). The p-toluene sulphonate formed rectangular plates, m.p. 76°, from ethanol. (Found: OMe, 9-8. C16H14O3ClS requires OMe, 9-9%).

The glucuronide of chloroquinol

(a) Isolation of the glucuronide. Chloroquinol (m.p. 105°) was found to be relatively toxic and was fed in doses of 100 mg./day to rabbits (2.5–3 kg.) so that 6 g. were fed to nineteen rabbits in 3 days. The urine (2100 ml.) was greenish brown in colour, did not reduce Benedict reagent and gave no colour with FeCl3; chloroquinol gave a transient blue-purple colour. The naphthoarsenic test on the urine and on an ether extract of it was intense. With 2,6-dichloroquinoline chloroquinol the urine gave a deep blue colour in the presence of NaHCO3; chloroquinol itself gives a red-brown colour under the same conditions.

The urine was saturated with (NH4)2SO4 and then exhaustively extracted with ethanol-ether (1:5). After treatment with charcoal and drying over anhydrous Na2SO4, the extract was evaporated to dryness under reduced pressure. The crude glucuronide was thus obtained as a dark syrup (12 g.).

(b) 3-Chloro-4-methoxyphenyl glucuronide. The above syrup was dissolved in 200 ml. ethanol and methylated repeatedly with ethereal diazomethane until the blue colour failed to appear in the 2,6-dichloroquinoline chloroimide test.
In this way 9 g. of syrupy 3-chloro-4-methoxyphenylglucuronic acid methyl ester was obtained. (Found: OMe, 16-6. C_{14}H_{17}O_{8}Cl requires OMe, 17-5 %.)

The whole of this ester was now dissolved in 30 ml of dry methanol and the solution saturated with NH_{2} at 0°. The mixture was kept overnight at room temperature in a stopped flask: a crystalline precipitate separated. After dilution with 20 ml of water and standing a further 6 hr. the crystals were filtered, washed with a little 50 % ethanol and dried in vacuo. The yield was 6 g. or 40 % of the dose. On recrystallization (charcoal) from water, 3-chloro-4-methoxyphenyl-β-D-glucuronamide monohydrate was obtained as cigar-shaped plates, m.p. 219–220° and [α]_{D}^{24} = 86-6° (c, 0-2 in acetone). It was soluble in ether and acetone, less soluble in ethanol and methanol and insoluble in cold water. (Found: C, 44.4; H, 5.0; N, 4.0; OMe, 9.0; H_{2}O, 4.9. C_{14}H_{17}O_{8}Cl.H_{2}O requires C, 44.4; H, 5.2; N, 4.0; OMe, 8.8; H_{2}O, 5.1 %.)

(c) Hydrolysis of the amide. The glucuronamide (1-8 g.) was hydrolysed by heating on a water bath for 4 hr. with 3 N HCl. The dark mixture was cooled and exhaustively extracted with ether. The ethereal extract now contained the chloromethoxyphenol and this was partly purified by extraction with 2 N NaOH, followed by acidification with dilute HCl and re-extraction with ether. After drying the extract with anhydrous Na_{2}SO_{4}, and treating with charcoal, the ether was evaporated and there remained a dark-brown gum (0-9 g.). This was distilled to give 0-38 g. of 3-chloro-4-methoxyphenol as a straw-coloured oil distilling at 150–170° (bath temp.)/6 mm. On standing the oil crystallized. It was identified as 3-chloro-4-methoxyphenol by the preparation from it of 3-chloro-4-methoxyphenyl benzolate (needles), m.p. and mixed m.p. 109–110°. (Found: OMe, 11.9. C_{14}H_{17}O_{4}Cl requires OMe, 11.8 %.) Also of 3-chloro-4-methoxyphenyl p-toluenesulphonate (plates), m.p. and mixed m.p. 95–96°. (Found: OMe, 9.8. C_{14}H_{17}O_{4}ClS requires OMe, 9.9 %.)

**DISCUSSION**

The glucuronide formed in the rabbit on feeding chloroquinol has been shown by the following series of reactions, which need no comment, to be 3-chloro-4-hydroxyphenylglucuronide:

\[ \text{Cl} \quad \text{OH} \quad \text{OH} \quad \text{Cl} \]

\[ \text{in vivo} \]

\[ \text{Cl} \quad \text{OH} \quad \text{OC}_{4}H_{10} \text{O}_{4} \]

\[ \text{Cl} \quad \text{OMe} \quad \text{OC}_{4}H_{10} \text{O}_{4} \text{CONH}_{2} \quad \text{OMe} \]

Thus it has now been shown that with 4-chlorocatechol, 4-chlororesorcinol (Dodgson & Williams, 1949) and chloroquinol, glucuronic acid conjugation is selective as regards the hydroxyl group undergoing conjugation. Furthermore, conjugation occurs on the hydroxyl group farthest away from the chlorine atom. This suggests that steric influences may be involved, and for this reason the approach of the enzyme system concerned in glucuronic acid conjugation to one hydroxyl is easier than to the other. Thus conjugation para to the chloro group is preferred to meta or ortho, and conjugation meta to the chloro group is preferred to ortho, when both are offered. Glucuronic acid conjugation does, however, occur in o-chlorophenol itself (Spencer & Williams, unpublished), but here, of course, only one hydroxyl is offered for conjugation.

In this work we have paid little attention to the sulphate conjugation, because the isolation, in a pure state, of the ethereal sulphates formed was not achieved. We did obtain evidence to show that, in the case of 4-chlorocatechol (Dodgson & Williams, 1949), the sulphate group was oriented in a similar way to the glucuronic acid group, and this may be generally true. Other work in this laboratory (Anderton, 1949) has suggested that the sulphate conjugation of chlorinated phenols may be correlated with the dissociation constant of the hydroxyl group. The extent of sulphate conjugation in chlorophenol has been shown by Williams (1938) to depend on the position of the chloro group relative to the hydroxyl, the conjugation being least with o-chlorophenol and greatest with p-chlorophenol. This observation can be correlated with dissociation constants and has now been extended to the polychlorinated phenols (see Table 1).

This table shows that the extent of conjugation of phenol with sulphuric acid is reduced by half its original value by an o-chloro group and to zero by two o-chloro groups, e.g. 2:6-dichloro-, 2:4:6-trichloro- and pentachloro-phenol. The last three phenols also have values of pK less than 7. Anderton, Smith & Williams (1948) have shown that phenols with pK less than 7 do not conjugate with sulphate in the rabbit.

Whether glucuronic acid conjugation can be correlated with dissociation constants is not yet clear. The constants of 4-chlorocatechol, 4-chlororesorcinol and chloroquinol do not appear to have been determined, but if one assumes that the strengths of the hydroxyl groups in these compounds are similar to those of the monochlorophenols, then in each case the least ionized hydroxyl group is the one which has conjugated with glucuronic acid.

**Glucuronic acid conjugation and its relation to the nature and position of hydroxyl groups in natural and other compounds**

A search of the literature has revealed four cases of the glucuronic acid conjugation of phenols similar to the ones studied here. Orcinol and phloroglucinol (IV) and (V) (Sera, 1913, 1914) give rise to monoglucuronides in the rabbit, but in these instances no orientation problem arises because the hydroxyl groups are symmetrically disposed in the molecule. 2:4-Dihydroxyacetophenone (VI) also gives rise to a monoglucuronide, but its structure was left un-
Table 1. **Ethereal sulphate conjugation of chlorophenols in the rabbit and dissociation constants**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (mg./kg.)</th>
<th>Percentage of dose conjugated with sulphate</th>
<th>$K_a \times 10^{10}$</th>
<th>pK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol</td>
<td>100*</td>
<td>18</td>
<td>1.7</td>
<td>9.77</td>
</tr>
<tr>
<td>Phenol</td>
<td>250*</td>
<td>19</td>
<td>32</td>
<td>8.49</td>
</tr>
<tr>
<td>2-Chlorophenol</td>
<td>325**</td>
<td>10</td>
<td>14</td>
<td>8.85</td>
</tr>
<tr>
<td>3-Chlorophenol</td>
<td>325**</td>
<td>22</td>
<td>6-6</td>
<td>9.18</td>
</tr>
<tr>
<td>4-Chlorophenol</td>
<td>325**</td>
<td>16</td>
<td>180</td>
<td>7.74</td>
</tr>
<tr>
<td>2,4-Dichlorophenol</td>
<td>163†</td>
<td>0</td>
<td>1600</td>
<td>6.80</td>
</tr>
<tr>
<td>2,6-Dichlorophenol</td>
<td>163†</td>
<td>10</td>
<td>180</td>
<td>7.74</td>
</tr>
<tr>
<td>2,4,5-Trichlorophenol</td>
<td>197-5‡</td>
<td>0</td>
<td>3800</td>
<td>6.42</td>
</tr>
<tr>
<td>Pentachlorophenol</td>
<td>206-5‡</td>
<td>0</td>
<td>55000</td>
<td>5.26</td>
</tr>
</tbody>
</table>

* Data quoted from Williams (1938).
† Equivalent to 100 mg. phenol.
‡ Equivalent to 250 mg. phenol.
$K_a$ Quoted from Murray & Gordon (1935).

defined by Nencki (1894) who described it as a 2- or 4-glucuronide. Our results suggest that Nencki's glucuronide is 4-glucuronosido-2-hydroxyacetophenone. A similar glucuronide of undetermined structure has been prepared by Salant & Bengis (1918) by feeding rabbits with benzeneazoresorculin (Sudan G35) (VII).

It appears to us that it is almost a general rule that when a compound containing more than one hydroxyl group capable (when occurring alone) of conjugating with glucuronic acid, is administered to an animal, only one is conjugated. As far as we are aware the only diglucuronide known is that of p-hydroxybenzoic acid (Quick, 1932). In this, one glucuronic acid residue is attached to the hydroxyl and the other to the carboxyl group. This has been isolated only from dog urine. In rabbits p-hydroxybenzoic acid gives rise to a monoglucuronide, the glucuronic acid being attached to the phenolic hydroxyl group (Hartles & Williams, 1948.) This rule also applies to the known naturally occurring glucuronides for they are all monoglucuronides. Thus euxanthic acid (euxanthone-7-glucuronide, Roberton & Waters, 1931), pregnanediol-3-monoglucuronide (Heard, Hoffman & Mack, 1944; Huebner, Overman & Link, 1944) and oestriol glucuronide (Cohen, Marrian & Odell, 1936; Grant & Marrian, 1948) are all monoglucuronides, although the aglycone in each case contains more than one hydroxyl group. In euxanthone both hydroxyl groups are phenolic, but it is the less sterically hindered 7-hydroxyl which is conjugated. In pregnanediol, both hydroxyls occur in secondary alcohol groups, but the one in the ring (the 3-hydroxyl) is selected for conjugation rather than the 20-hydroxyl in the side chain. Oestriol offers three hydroxyls for conjugation, one phenolic and two in secondary alcohol groups, but the phenolic group is certainly free in oestriol monoglucuronide (Cohen et al. 1936) and the glucuronic acid is attached to the 16- or 17-hydroxyl group.

Thus it becomes clear from our work on the chlorinated dihydric phenols and from the natural glucuronides cited above that in the selection of the hydroxyl group for conjugation both the position and the nature (i.e. whether it is phenolic or occurs in a primary, secondary or tertiary alcohol) of the hydroxyl group is involved. Although some examples are known of the conjugation of dihydroxy compounds containing two different kinds of hydroxyls, much more information is needed before definite rules can be drawn up for ten different combinations of the four kinds of hydroxyls mentioned above taken two at a time can occur.

Examples of some of these combinations are already known: (1) When two phenolic hydroxyl groups occur in a compound, then, if they are not equivalent, the one which is least sterically hindered is conjugated, e.g. euxanthone, chloroquinol, etc. (2) An example of a compound containing two differently oriented secondary hydroxyl groups is 1:2-dihydro-1:2-dihydroxyantracene which is excreted as a monoglucuronide when anthracene is fed to rabbits (Boyland & Levi, 1936). Here the 1-hydroxyl is selected for conjugation. Pregnanediol is another but different example. (3) In oestriol monoglucuronide a secondary hydroxyl is conjugated rather than the phenolic hydroxyl. (4) An example of a substance containing one primary and one secondary hydroxyl group is propylene glycol which forms a monoglucuronide in rabbits. Miura (1911) suggests, without proof, that the glucuronic acid is attached to the primary and not the secondary hydroxyl. (5) p-Menthane-1:8-diol contains a secondary and a tertiary hydroxyl group, and Kuhn & Löw (1938) have shown that in the monoglucuronide formed when the diol is fed to rabbits, the
uronic acid residue is attached to the secondary and not the tertiary hydroxyl.

Although the evidence as yet is very meagre, it does suggest that when a compound containing two hydroxyls of different nature is offered for conjugation, then a primary hydroxyl is preferred to a secondary and a secondary to a tertiary or phenolic hydroxyl.

SUMMARY

1. A study of the conjugation of chloroquinol with glucuronic acid has been carried out in the rabbit.
2. Chloroquinol forms a monoglucuronide which was isolated and characterized as 3-chloro-4-methoxyphenylglucuronidamide. The structure of this glucuronidamide has been proved.
3. It has been proved that glucuronic acid conjugates with the hydroxyl group of chloroquinol farthest away from the chloro group.
4. 2- and 3-Chloro-4-methoxyphenols and some of their derivatives have been synthesized and used as reference compounds.
5. The results are discussed in relation to the orientation of glucuronic acid conjugation in some natural glucuronides.

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REFERENCES