3. The stimulant effects of the synthetic hypoglycaemic agents Synthalin and phenethyl diguanide are not affected by insulin antiserum.

4. The significance of these findings is discussed and it is suggested that such insulin antiserum could be used to aid in the identification of insulin in extracts from biological materials.

I wish to thank Boots Pure Drug Co. Ltd., Nottingham, for generous supplies of the insulin preparations and the phenethyl diguanide used in these experiments, and especially Mr S. S. Randall who kindly extracted the insulin from human pancreases; Dr A. B. Stavitsky of Western Reserve University, Cleveland, U.S.A., for a sample of rabbit serum; Dr R. H. W. Britton of Hoechst Pharmaceuticals Ltd., Slough, for samples of tolbutamide and carbutamide; and Dr J. D. Pearson of this Medical School for the ^{125}I -labelled bovine insulin. I would also like to thank Professor R. H. S. Thompson for his continued encouragement and helpful advice and Miss Beryl Hartman and Mr K. G. Kilbourn for skilled technical assistance.

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Studies in Detoxication

78. GLUCURONIDE AND ETHEREAL SULPHATE FORMATION IN HENS*

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(Received 28 October 1958)

The fate of foreign organic compounds in the domestic fowl has not been extensively investigated. Most of the earlier studies were concerned with the synthesis of ornithuric acid (N^2N^5 -dibenzoylornithine, first discovered by Jaffe in 1877) because the fowl appeared to differ from most other species, since it conjugated aromatic acids with ornithine rather than with glycine. Preliminary work in this Laboratory with ^{14}C benzoic acid suggests that ornithuric acid formation may account for nearly half of moderate doses of benzoic acid. References to other reactions of foreign compounds in hens are few, e.g. acetylation (Crowdle & Sherwin, 1923; Shaffer & Beiter, 1950),

methylation (Hoshiai, 1909; Takahashi, 1927; Tamura, 1924), oxidation and reduction (Crowdle & Sherwin, 1923). Sperber (1947) found that phenols injected into hens caused an increase in the excretion of ethereal sulphates and of glucuronic acid derivatives as measured by the naphtharesorcinol reaction, but injected benzoic acid, *p*-aminobenzoic acid and chloral did not increase glucuronic acid excretion. This suggested that hens could form the ether glucuronides of phenols, but not the ester glucuronides of aromatic acids. Wu Chang & Johnson (1957), however, claim to have detected by paper chromatography nicotinoyl glucuronide, an ester glucuronide, in the urine of chicks giving nicotinamide or nicotinic acid. In the present work we have shown, by isolation of the

* Part 77: McIsaac, Parke & Williams (1958).

conjugates, that the domestic fowl can synthesize appreciable amounts of both ether and ester glucuronides and of ethereal sulphates.

MATERIALS AND METHODS

Diet and animals. The hens used were Rhode Island Red crossed with Light Sussex, weighing 2.5–3.9 kg. They were maintained on 150 g. of mash/day (Superbra battery, intensive mash, Poultry Association of Great Britain). Compounds were administered to the hens by crop tube. Usually the compounds were fed at a dose level of 2 mmole/kg., dissolved or suspended in 25 ml. of water to which a knife-point of bile salt had been added.

Collection of excreta and analytical methods. In studies with hens one of the main difficulties is the collection of urine and of faeces separately. We found that the determination of glucuronic acid and ethereal sulphate in mixed urine and faeces was completely unsatisfactory. Various techniques have been described for the separation of urine from faeces. Surgical operations for separating the rectum from the cloaca and for exteriorizing the ureters have been criticized as leaving the hen in an abnormal condition. Cannulation of the ureters has been used, but this is difficult since the ureters are so small and difficult to locate. Hart & Essex (1942) used a special cannula fitted with a glass ball to block the rectum, but this can be used only for periods of less than 2 hr., which were insufficient for our experiments. We found, however, that if food was withheld

Table 1. *Glucuronide excretion by hens given borneol*

On days 1, 3, 6, 7, 8, 10 and 12 food was given to the hens. DL-Borneol (304 mg./kg.) was given on day 4.

Day	Hen 15 (2.7 kg.)		Hen 16 (2.7 kg.)		Hen 17 (2.4 kg.)	
	Glucuronic acid output (mg.)	Urine vol. (ml.)	Glucuronic acid output (mg.)	Urine vol. (ml.)	Glucuronic acid output (mg.)	Urine vol. (ml.)
2	3.6	22	2.7	13	6.2	31
4	205.0	100	198.0	40	174.0	68
5	58.5	90	76.7	26	5.1	17
9	6.8	109	11.2	20	6.9	15
11	5.8	92	1.2	19	14.0	35
13	4.0	80	3.6	17	3.1	8
Average normal value	5.0	—	4.7	—	7.5	—
Excess after borneol	253.5	—	265.3	—	166.5	—
Percentage of dose conjugated with glucuronic acid	25	—	26	—	18	—

Table 2. *R_F values of some glucuronides and ethereal sulphates*

R_F values are for descending chromatography on Whatman no. 4 paper. Solvent mixtures: *A*, in butanol–acetic acid–water (4:1:2, by vol.) run for 6 hr.; *B*, butanol–benzene–pyridine–water (5:1:3:3, by vol.) run for 4 hr.; *C*, propanol–aq. NH₃ soln. (sp.gr. 0.88) (7:3, by vol.) run for 5 hr.; *D*, butanol–acetic acid–water (4:1:5, by vol.) run for 6 hr. In the times quoted the solvent front moved about 30 cm. from the origin.

Sprays used: *a*, aq. 1% naphtharesorcinol followed by heating at 110° in an atmosphere of HCl gas, which gave dark-blue spots with glucuronic acid and glucuronides; *b*, snail digestive juice (diluted with 10 vol. of water; see Jarrige & Henry, 1952) followed by incubation at 37° for at least 1 hr. between glass plates, and then spraying with aq. 0.1% 2:6-dichloroquinone chloroimide followed by saturated NaHCO₃, which gave blue spots with certain phenolic glucuronides and sulphates. *m*-Aminophenylsulphate was detected as a red spot, which turned brown on keeping, by spraying successively with 0.1% NaNO₂ in 0.2N-HCl, 1% ammonium sulphamate and 0.5% α-naphthylamine in diluted ethanol, allowing the paper to dry between each spray. Illumination of the paper with u.v. light from a low-pressure mercury arc lamp (Hanovia Chromatolite) revealed *o*-methoxybenzoic acid and its glucuronide as purple fluorescent spots, and glucurone and glucuronic acid quenched the background fluorescence of the paper.

Solvent mixture	<i>R_F</i> values			
	<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>
Compound				
Glucuronic acid*	0.28	—	0.07	—
Glucurone	0.49	0.64	0.07 0.23	—
Bornylglucuronide	0.91	0.59	0.76	—
<i>o</i> -Methoxybenzoic acid	—	0.65	—	0.94
<i>o</i> -Methoxybenzoylglucuronide	—	0.26	—	0.58
Phenylglucuronide	—	0.22	0.45	—
Phenylsulphate†	—	0.60	0.84	—
<i>m</i> -Aminophenylsulphate†	0.45	0.35– 0.41	0.65	—

* Applied to the paper as the sodium salt.

† Applied as the potassium salt.

from the hen for 24 hr., a greenish fluid containing little or no solid faeces was excreted. This fluid, which we assumed to be mainly urine, could be used satisfactorily for the determination of glucuronic acid and ethereal sulphate.

The technique eventually adopted was to give the hen a double ration of food (300 g.) on one day and none on the second day. Most of the food was eaten on the first day and the excreta for this day were discarded; the excreta for the second day contained little or no solid matter and were collected and analysed. When foreign compounds were administered, food was withheld for 48 hr. and quantitative estimations were carried out on the urine excreted on these 2 days. Glucuronic acid was determined according to Paul (1951; see Mead, Smith & Williams, 1958) and under the above conditions the average normal excretion of glucuronic acid was 7.5 mg./day (range 1.2–18.5 mg.) (28 determinations on six hens). Typical quantitative results are given in Table 1. Determination of ethereal sulphate was carried out by the method of Folin (1905–06). The turbidimetric method of Sperber (1948) gave completely unsatisfactory results. The average ethereal sulphate output was 3.8 mg. of SO_3 /day (range 0.4–8.5 mg.) (22 determinations on six hens).

Chromatography. R_F values for the compounds used are shown in Table 2.

ISOLATION AND DETECTION OF GLUCURONIDES AND ETHEREAL SULPHATES

Glucuronide of chloroxylenol

From rabbits. Two rabbits were given, by stomach tube, 2 g. each of chloroxylenol (4-chloro-3,5-dimethylphenol) and their urine was collected for 24 hr. The glucuronide was separated as the basic lead salt (Kamil, Smith & Williams, 1951). After removal of the lead, the aqueous solution of the glucuronide was concentrated *in vacuo* at 40–50° to a jelly which did not crystallize. It was therefore dried *in vacuo* and acetylated with acetic anhydride containing one drop of 60% perchloric acid. On diluting with water, 4-chloro-3,5-dimethylphenyl triacetylglucosiduronic acid (0.3 g.) separated as white plates, m.p. 201–202° after recrystallization from methanol; $[\alpha]_D^{21} - 22 \pm 1^\circ$ in ethanol (c, 1) (Found: C, 52.15; H, 5.35. $\text{C}_{20}\text{H}_{25}\text{O}_{10}\text{Cl}$ requires C, 52.35; H, 5.05%). In another experiment the glucuronide fraction was prepared from the urine of a rabbit which had been given 1.5 g. of chloroxylenol. The dried glucuronide fraction prepared as above was first methylated with diazomethane and then acetylated with pyridine and acetic anhydride (see Kamil *et al.* 1951). On diluting the acetylation mixture with water, methyl (4-chloro-3,5-dimethylphenyl tri-O-acetyl-D-glucosid)uronate (0.5 g.) separated as colourless needles, m.p. 176° after recrystallization from methanol, and $[\alpha]_D^{21} - 30 \pm 1^\circ$ in CHCl_3 (c, 1) (Found: C, 53.7; H, 5.6. $\text{C}_{21}\text{H}_{25}\text{O}_{10}\text{Cl}$ requires C, 53.3; H, 5.3%). The ester (100 mg.) in 4N- H_2SO_4 (20 ml.) was boiled under reflux for 2 hr. and then the mixture was steam-distilled. From the distillate, chloroxylenol (20 mg.) was recovered, m.p. and mixed m.p. 114°.

An attempt was also made to prepare free chloroxylenol glucuronide. The 24 hr. urine of two rabbits, each dosed with 2 g. of chloroxylenol, was brought to pH 3–4 by adding 2N- H_2SO_4 , and then continuously extracted with ether for 24 hr. Crystals (0.1 g.) separated in the ether and

appeared to be chloroxylenol glucuronide (m.p. 202–206° and $[\alpha]_D^{20} - 56^\circ$ in water) but recrystallization from a variety of solvents gave gels. On acetylation, however, the triacetyl acid (m.p. 202° and $[\alpha]_D^{20} - 22^\circ$ in ethanol) was obtained.

From hens. Two hens which had been starved overnight were each given 2 g. of chloroxylenol in water by crop tube. The mixed urine and faeces were collected for the next 2 days. The first day's excreta were centrifuged. The supernatant fluid was brought to pH 2–3 with 2N- H_2SO_4 and continuously extracted with ether for 24 hr. The extract was dried over anhydrous Na_2SO_4 and then treated with an excess of diazomethane in ether. The ether was evaporated and the dry residue treated with acetic anhydride (4 ml.) and 1 drop of 60% perchloric acid. When the reaction had subsided, the mixture was diluted with water and the crystals (0.38 g.) which separated were collected. After recrystallization from ethanol, the white needles obtained were identified as methyl (4-chloro-3,5-dimethylphenyl tri-O-acetyl-D-glucosid)uronate, m.p. and mixed m.p. 175–176° and $[\alpha]_D^{20} - 31.6^\circ$ in CHCl_3 (c, 0.9).

m-Cresyl glucuronide

From rabbits. m-Cresol (2 g.) in water was administered to a rabbit by stomach tube. The 24 hr. urine was collected and the glucuronide fraction separated by the lead acetate method (Kamil *et al.* 1951). The glucuronide gum was methylated and acetylated to give 3.5 g. of crude methyl (m-cresyl tri-O-acetylglucosid)uronate. The compound recrystallized from ethanol and light petroleum (b.p. 100–120°) had m.p. 109° and $[\alpha]_D^{20} - 35^\circ$ in CHCl_3 (c, 1) (Found: C, 56.7; H, 5.8; OMe, 7.6. Calc. for $\text{C}_{20}\text{H}_{24}\text{O}_{10}$: C, 56.6; H, 5.7; OMe, 7.35%). Kamil *et al.* (1951) give m.p. 93–94° and $[\alpha]_D - 25.6^\circ$, but their compound appears to have been contaminated with a second glucuronide which occurs to the extent of about 10% of the glucuronide fraction and is being investigated by one of us (D.R.).

From hens. m-Cresol (1 g.) in aqueous suspension was administered orally to a hen (3.5 kg.). Food was withheld overnight and in the morning 100 ml. of thick green urine was collected. The urine was diluted with an equal volume of acetone and the precipitate which formed was discarded. The acetone was evaporated *in vacuo* and the residual solution subjected to the lead acetate procedure (Kamil *et al.* 1951). The glucuronide was obtained as a neutral gum which was dissolved in water (20 ml.) and then treated with Zeo-Karb 225 (acid form; 0.2 g.). The resin was filtered off and the filtrate evaporated *in vacuo* to an acid gum (0.2 g.). The gum was methylated and acetylated to yield 0.28 g. of methyl (m-cresyl tri-O-acetylglucosid)uronate, m.p. and mixed m.p. 109°, and $[\alpha]_D^{20} - 31.5^\circ$ in CHCl_3 (c, 2) after several recrystallizations from ethanol and light petroleum (b.p. 100–120°).

Benzoyl glucuronide

From dogs. Methyl (benzoyl glucosid)uronate had been prepared (cf. Quick, 1926) from a sample of benzoyl glucosiduronic acid obtained by Pryde & Williams (1933) by feeding dogs with benzoic acid. The ester, which reduced Benedict's reagent on heating, had m.p. 186° and $[\alpha]_D^{21} - 16.1^\circ$ in methanol (Found: C, 53.5; H, 5.1; OMe, 9.3. Calc. for $\text{C}_{14}\text{H}_{16}\text{O}_8$: C, 53.8; H, 5.2; OMe, 9.9%).

Table 3. Absorption spectra in ethanol of benzoic acid and its conjugates

	$\lambda_{\max.}$		Corresponding $\epsilon_{\max.}$	
	229	273	12 000	860
Benzoic acid				
Benzoyl glucosiduronic acid (from hen urine)	230.5	275	14 300	1270
Methyl (benzoyl glucosid)uronate (from dog urine)	230	275	13 900	1070
Methyl (benzoyl glucosid)uronate (from hen urine)	230	275	13 300	1110
Hippuric acid	226	~269	10 750	About 700

From hens. Two hens, deprived of food overnight, were each given 1 g. of benzoic acid in water by crop tube. The urine was collected for 18 hr., brought to pH 2–3 with 2*N*-H₂SO₄ and continuously extracted with ether for 16 hr. The extract was dried over anhydrous Na₂SO₄ and evaporated to a thick syrup, which was then taken up in CHCl₃ (150 ml.). This solution was kept overnight at 0° and a white amorphous solid (150 mg.) separated. From its solution in the least volume of ethanol benzoyl glucosiduronic acid was precipitated by light petroleum (b.p. 40–60°) as a white powder, m.p. 180° and $[\alpha]_D^{20}$ –25.6° in water (c, 0.7), which readily reduced Benedict's reagent and gave an intense naphtharesorcinol reaction. Goebel (1938) gives m.p. 184–185° and $[\alpha]_D$ –26.8° in water. The absorption spectrum of the acid was similar to that of benzoic acid (see Table 3).

The acid (30 mg.) was converted into its methyl ester with ethereal diazomethane at –15°. This ester reduced Benedict's reagent and, after recrystallization from water, it was obtained as white needles, m.p. 191–193° and mixed m.p. 190–191°, $[\alpha]_D^{21}$ –17.5° in methanol (c, 0.4). Its absorption spectrum was identical with that of the ester obtained from dog urine (see Table 3). A small amount (15 mg.) was acetylated with acetic anhydride (1 drop) and pyridine (1 drop) to yield methyl (benzoyl tri-*O*-acetylglucosid)uronate, m.p. 143–144°. Goebel (1938) gives m.p. 145°.

o-Methoxybenzoyl glucuronide

From rabbits. *o*-Methoxybenzoic acid (2 g.) was administered to each of four rabbits and the urine was collected for the subsequent 24 hr. This urine reduced Benedict's reagent on warming and gave an intense naphtharesorcinol reaction. The glucuronide was separated by the lead acetate procedure of Kamil *et al.* (1951). It was obtained as white needles (5 g.) and was purified by repeated recrystallization from ethanol. The compound separated from this solvent as white needles containing one molecule of ethanol of crystallization, m.p. 121–122° and $[\alpha]_D^{20}$ –34.5±2° in water (c, 1). The *o*-methoxybenzoyl glucosiduronic acid ethanolate reduced Benedict's reagent on warming (Found: C, 51.2; H, 5.6; (OMe + OEt)/2, 21.5. C₁₄H₁₆O₉, C₈H₈O₄ requires C, 51.35; H, 5.9; 1 OMe + 1 OEt, 20.3%). Methyl (*o*-methoxybenzoyl tri-*O*-acetyl-*D*-glucosid)uronate was prepared by methylation and acetylation of the preceding ethanolate and formed white needles, m.p. 138° and $[\alpha]_D^{20}$ –7±2° in CHCl₃ (c, 1.5) (Found: C, 54.15; H, 5.5; OMe, 13.3; C₂₁H₂₄O₁₂ requires C, 53.8; H, 5.2; OMe, 13.2%).

From hens. Three hens were given about 0.8 g. each of *o*-methoxybenzoic acid in water by crop tube. Paper chromatography of the urine in two solvents showed *o*-methoxybenzoyl glucuronide to be present. Attempts to isolate the glucuronide or a derivative were not successful.

m-Aminophenylsulphate

Preparation of 5-aminoacridine m-aminophenylsulphate. This salt was prepared by adding an excess of hot saturated aqueous solution of 5-aminoacridine hydrochloride to a saturated aqueous solution of potassium *m*-aminophenylsulphate (prepared according to Smith, 1955). The salt immediately separated from the solution as yellow needles, m.p. 240° after several recrystallizations from hot water containing 10–20% of ethanol (Found: SO₄, 24.8; C₆H₄NSO₄[–], 49.3; C₁₃H₁₀N₂, 50.8. C₁₉H₁₇N₃SO₄ requires SO₄, 25.05; C₆H₄NSO₄[–], 49.1; C₁₃H₁₀N₂, 50.6%). Sulphate was determined turbidimetrically (Sperber, 1948), *m*-aminophenylsulphate colorimetrically (Smith, 1955) and 5-aminoacridine fluorimetrically.

m-Aminophenylsulphate from hen excreta. Two hens (each weighing 2 kg.) were given 0.5 g. of *m*-aminophenol each. The phenol was suspended in water containing a little bile salt and administered by crop tube in the morning, the birds having last been given food the previous evening. The hens were given water *ad lib.* but no food for 24 hr. The excreta produced in this time were mainly liquid; they were pooled and centrifuged. The supernatant liquid was saturated with K₂CO₃ and then extracted with acetone (4 × 250 ml.) which had been kept in contact with saturated K₂CO₃ solution. The extract was filtered and evaporated under reduced pressure to 20 ml. The residue was transferred to a beaker and treated with 5–10 ml. of hot saturated aqueous 5-aminoacridine hydrochloride solution until the yellow precipitate flocculated. After being kept at 0° overnight, the yellow crystals were collected and recrystallized (charcoal) several times from hot water containing 10–20% of ethanol. The crystals were identified as 5-aminoacridine *m*-aminophenylsulphate, m.p. and mixed m.p. 239–240° (yield 0.61 g. or 17.4% of the dose) (Found: SO₄, 24.3; C₆H₄NSO₄[–], 51.0; C₁₃H₁₀N₂, 50.5%). The potassium *m*-aminophenylsulphate prepared from this salt showed *R_F* values in three solvents identical with those of the synthetic sample (see Table 2).

Phenylglucuronide and phenylsulphate were detected by paper chromatography in the urine of phenol-fed hens. The formation of bornylglucuronide after administration of (±)borneol was also shown to occur.

Table 4. *Glucuronic acid conjugation in hens*

Compounds were administered orally (dose 2 m-mole/kg.). Figures quoted are the average for three animals, the range being in parentheses.

Compound fed	Percentage of dose excreted conjugated			
	By hens		By rabbits	
	As glucuronide	As ethereal sulphate	As glucuronide	As ethereal sulphate
Benzoic acid	36 (28-47)	—	15*	—
(±)Borneol	23 (18-26)	2 (1-3)†	26 (24-29)	—
Chloroxylenol	27 (23-31)	—	53 (43-71)	21 (17-27)
<i>o</i> -Methoxybenzoic acid	26 (21-34)	1 (0.5-1.5)†	67 (62-75)	—

* Quoted from Bray, Humphris, Thorpe, White & Wood (1955).

† Probably not significant.

RESULTS AND DISCUSSION

Quantitative results on the glucuronic acid output of hens dosed with two aromatic acids, a phenol and a terpene secondary alcohol, are given in Table 4, which shows that glucuronic acid conjugation is a mechanism of importance in the hen since it can account for nearly one-third of the dose of these compounds. Furthermore, hens can form glucuronides of both the ether and ester type. Chloroxylenol and *o*-methoxybenzoic acid are more extensively conjugated with glucuronic acid in rabbits than in hens, but with benzoic acid the reverse seems to be true. In the rabbit, glycine conjugation is the major mechanism by which benzoic acid is metabolized and it can account for 80% or more of the benzoic acid fed (Bray, Humphris, Thorpe, White & Wood, 1955). Glycine conjugation of benzoic acid does not occur in hens and is replaced by ornithine conjugation, which can account for up to 50% of a dose of 2 m-mole of benzoic acid/kg. (B. C. Baldwin, D. Robinson & R. T. Williams, unpublished work). Borneol seems to be equally well conjugated with glucuronic acid in the hen and the rabbit.

Sulphate conjugation of phenols also occurs in hens in which *m*-aminophenol is conjugated with sulphate to at least 17-18% of the dose (0.25 g./kg.) based on isolation; the sulphate conjugation of *m*-aminophenol in rabbits is 15% of the dose (1 g./rabbit) (Bray, Clowes & Thorpe, 1952).

SUMMARY

1. Phenol, *m*-cresol, 4-chloro-3:5-dimethylphenol, borneol, benzoic acid and *o*-methoxybenzoic acid when fed to hens are excreted as conjugated glucuronic acids. Glucuronic acid conjugation is a mechanism of importance in hens and can account for about a third of moderate doses of the last four of these substances.

2. The glucuronides of *m*-cresol, chloroxylenol

and benzoic acid were isolated from chicken urine as crystalline derivatives and were compared with the same derivatives isolated from rabbit or dog urine.

3. Phenols are conjugated with sulphate in hens. The ethereal sulphate of *m*-aminophenol was isolated from hen urine as the 5-aminoacridine salt.

The work was entirely supported by a grant from Dr Salsbury's Laboratories, Charles City, Iowa.

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