Tetrahedron, 1959, Vol 7. pp. 252 to 256. Pergamon Press Ltd, Printed in Northern Ireland

THE OCCURRENCE OF PROTOCATECHUIC ACID AND ITS 4-O-β-D-GLUCOSIDE IN *BLATTA* AND *PERIPLANETA*

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(*Received* 29 *April* 1959)

Abstract—(1) The left colleterial glands of two species of cockroach secrete a β-glucoside of protocatechuic acid, which can be identified readily by chromatography and ultra-violet spectroscopy.

(2) The compound is identical with 4-O-β-D-glucosidoprotocatechuic acid synthesized from 3-O-acetylprotocatechuic acid and 2:3:4:6-tetra-O-acetyl-D-glucosyl-α-bromide.

(3) The ultra-violet spectra of the synthetic and naturally occurring glucosides have been correlated with those of known substituted hydroxybenzoic acids.

THERE are few recorded instances of aromatic glucosides participating in animal metabolic processes. In insects, however, two glucosidic pigments have been described. Hollande[1] showed that a violet glucosidic pigment was present in the fat body of the larva of the beetle *Cionus*; and Brown *et al.*[2] have shown that protoaphins, found in the haemolymph of aphids, are glucosides. In addition, aromatic glucosides have been detected[3] as transient metabolites, being the form in which phenols are detoxified by insects. In a previous publication,[4] evidence was presented for the existence of a β-glucoside of protocatechuic acid in the colleterial or sexual accessory glands of the cockroaches *Blatta orientalis L.* and *Periplaneta americana* (L.)

Both these animals lay eggs in batches of 16 surrounded by a hard brown capsule, known as the ootheca, the walls of which are composed of a quinone-tanned protein, sclerotin. This material, which is of widespread occurrence, particularly in the exoskeletons of invertebrates, was shown by Pryor[5] to be a protein hardened by an *o*-quinonoid substance. Subsequently, Pryor *et al.*[6] isolated protocatechuic acid, the presumed precursor of the quinone, from extracts of cockroach oothecae. The oothecal wall and fluid contents are derived from secretions of the two colleterial glands. The larger left gland contains an abundance of a white, sticky, proteinaceous material; to the small right gland was ascribed[5] the function of secreting protocatechuic acid. Pryor also showed that when isolated tissue of the left gland was incubated with that of the right gland, the contents of the lumen of the former became brittle, brown, and insoluble in dilute mineral acids thus resembling sclerotin. A similar phenomenon was observed[6] when the left gland was incubated with synthetic protocatechuic acid. The presence of this acid in the oothecal fluid has now been confirmed and in addition the fluid is found to contain glucose. However, chromatographic analysis of aqueous or ethanolic extracts of either left or right colleterial failed to reveal the presence of the free acid.[4] When extracts of the left gland

[1] A. C. Hollande, *Arch. Zool. exp. gen.* (Notes et Revues), **51**, 53 (1913).
[2] B. R. Brown, T. Ekstand, A. W. Johnson, S. F. MacDonald and A. R. Todd, *J. Chem. Soc.* 4925 (1952).
[3] C. M. Myers and J. N. Smith, *Biochem. J.* **56**, 498 (1954).
[4] P. C. J. Brunet and P. W. Kent, *Proc. Roy. Soc.* B **144**, 259 (1955).
[5] M. G. M. Pryor, *Proc. Roy. Soc.* B **128**, 378 (1940).
[6] M. G. M. Pryor, P. B. Russell and A. R. Todd, *Biochem. J.* **40**, 627 (1946).

(in contrast to the right gland) were hydrolysed with dilute sulphuric acid, the protocatechuic acid and glucose could be readily detected. Further investigation showed that these two substances could be also obtained by the action of yeast β-glucosidase on extracts of the left gland. We have now synthesized the 4-O-β-D-glucoside of protocatechuic acid and have shown its presence in the left gland of both species of cockroach.

DISCUSSION

The finding of substantial amounts of protocatechuic acid in the oothecal fluid of *Blatta* and *Periplaneta* and in other sclerotized parts of the animals raises the question of its biochemical role, and the form in which such a reactive compound can be stored in the body. It is clear from Pryor's results[5] that a source of this very reactive acid is located in the colleterial glands, but the present investigation has shown that the free acid is not detectable in either right or left colleterial gland. However, when the secretion of the left gland was treated with yeast β-glucosidase, or was subjected to acidic hydrolysis, then the free acid together with D-glucose was found, indicating that the left colleterial gland probably secreted a β-D-glucoside of the acid. The finding that this glucoside, obtained chromatographically from aqueous ethanolic extracts of the left gland was laevorotatory, supported this view.

On the other hand no such substance was present in the right colleterial gland, the secretion of which was found actively to cleave added β-glucosides such as salicin or cellobiose, as well as the glucoside of the left gland. This gland was evidently implicated in the formation of free protocatechuic acid.

The chemical structure of the glucoside of the left gland was then further investigated. Since the glucoside is not readily oxidized in air under alkaline conditions, and gives tests only for monophenols and not for diphenols, it is evident that the glucose moiety is attached to the hydroxyl group at C-3 or C-4. The location of the glucosidic linkage at C-4 has been established by definitive chemical synthesis, and by ultra-violet spectroscopic examination.

The 4-O-β-D-glucoside or protocatechuic acid was synthesized by conventional methods as follows. 3-O-Acetylprotocatechuic acid, prepared by the method of Lesser and Gad,[7] provided a protected starting material. This substance had a strong absorption peak at λ 251 mμ (in ethanol), resembling that of vanillic acid (λ_{max} 260 mμ) and *p*-hydroxybenzoic acid (λ_{max} 251 mμ). The acetyl-derivative was coupled with 2:3:4:6-tetra-O-acetyl-D-glucosyl-α-bromide in the presence of quinoline and silver oxide.[8] This method gave yields markedly superior to several others investigated.[9,10]

Yields were still further improved by starting with 3-O-benzoylprotocatechuic acid and proceeding by an analogous series of reactions. The acetylated glucoside was a crystalline solid ($[\alpha]_D^{19}$-40°), which on deacetylation gave a crystalline product. This had an identical R_F value with the naturally-occurring glucoside, and it was hydrolysed by yeast, and right gland β-glucosidase. In water, the synthetic material had an ultra-violet spectrum identical with the natural product. Further evidence in favour of the 4-O-substituent was obtained by examination of ultra-violet spectra in ethanol,

[7] R. Lesser and G. Gad, *Ber. Dtsch. Chem. Ges.* **59**, 233 (1925).
[8] A. Robertson and R. B. Waters, *J. Chem. Soc.* 2729 (1930).
[9] T. H. Bembry and G. Powell, *J. Amer. Chem. Soc.* **64**, 2419 (1942).
[10] B. Helferich and W. Reischel, *Liebigs Ann.* **533**, 278 (1938).

in M/50 and in M/500 sodium ethoxide. The changes of λ_{min} and λ_{max} in these conditions agree with figures for known[11] substituted hydroxybenzoic acids (Table 1). Glycosidation or methylation of an hydroxyl group in general induces little change in position of λ_{max} (usually a slight hypsochromic shift).

TABLE 1. ULTRA-VIOLET SPECTRA OF 4-O-β-D-GLUCOSIDE OF PROTOCATECHUIC ACID AND OTHER SUBSTITUTED HYDROXYBENZOIC ACIDS

	Solvent	max	min	max
Natural glucoside from left gland	EtOH	238 mμ	266 mμ	290 mμ
	M/50 EtONa	255	275	310
	M/500 EtONa	—	272	292
Synthetic glucoside	EtOH	239	265	289
	M/50 EtONa	241	275	308
	M/500 EtONa	—	274	295
Vanillic acid†	EtOH	260	280	292
	M/50 EtONa	—	245	295
	M/500 EtONa	—	245	295
Isovanillic† acid	EtOH	257	273	293
	M/50 EtONa	257	280	308
	M/500 EtONa	—	272	290

† Values from Dr. T. Swain.

The evidence indicates thus that protocatechuic acid is stored in the form of its 4-O-β-D-glucoside by the left gland which also secretes the protein constituents of sclerotin and a polyphenol oxidase.[12] The right gland secretes a β-glucosidase such that in the intermingled secretions in the genital vestibulum, free protocatechuic acid is liberated. This is oxidized by the polyphenol oxidase to an *o*-quinone which condenses with the available protein ("tanning reaction") to form sclerotin, the resilient and stable material of which the egg-capsule is made.

EXPERIMENTAL

Paper chromatography. Chromatograms were prepared by descending elution on Whatman No. 1 paper using (except where otherwise stated) n-butanol (40%), acetic acid (10%), water (50%) (v/v). Phenols were detected by spraying chromatograms with the following reagents, (i) 1% (w/v) ferric chloride mixed with an equal volume of 1% (w/v) potassium ferricyanide,[13] (ii) 1% (w/v) ferric chloride which gives a green colour with *o*-dihydroxyphenols, becoming pink in NH_3 vapour, (iii) 'chromaffine reagent',[14] i.e. 5% (w/v) solution of KIO_3. This solution gives a yellow or brown colour with dihydroxyphenols. (iv) Pauly's diazo solution[15] i.e. 0·2% (w/v) sulphanilic acid in 0·5

[11] G. H. Mansfield, T. Swain and G. G. Nordstrom, *Nature, Lond.* **172**, 23 (1953).
[12] P. C. J. Brunet, *Quart. J. Micr. Sci.* **93**, 47 (1952).
[13] G. M. Barton, R. S. Evans and J. A. F. Gardner, *Nature, Lond.* **170**, 249 (1952).
[14] L. Lison, *Histochimie Animale* Gautrier-Villars, Paris (1936).
[15] V. Erspamer and G. Boretti, *Arch. Int. Pharmacodyn.* **88**, 295 (1951).

N–HCl (10 vols) mixed with 1% (w/v) sodium nitrite (1 vol) (v) Dihydroxyphenols and other reducing substances were also detected with 5% (w/v) silver nitrate to which 0·88 ammonia solution was added to redissolve the initial precipitate.

Periplaneta americana and *Blatta orientalis* were cultured at 25°. Colleterial glands were dissected under saline from mature adults killed by decapitation. Oothecae were collected at weekly intervals from flower pots filled with moist sand which proved to attract ovipositing females.

Yeast β-glucosidase was supplied by L. Light & Co. Ltd., Colnbrook, Bucks.

Microanalyses were performed by Dr. A. Bernhardt of Mulheim, Ruhr.

Examination of oothecal fluid

Oothecae were carefully punctured and squeezed in a small screw device so as to express the fluid contained in the space between the eggs and the walls. From 1000 oothecae, 12·8 ml fluid were collected. This had a pink colour, and contained 3·75% (w/v) of dissolved matter; the pH value was 5·6. It gave immediate positive chromaffine and silver nitrate reactions for *o*-dihydroxyphenols and a strong ultra-violet light absorption at 253 mμ. Chromatographic examination of the fluid revealed one principal phenolic constituent (R_F 0·86) identical with protocatechuic acid. Elution of the excised chromatographic spot with 5 ml water gave a solution with absorption spectrum identical with that of protocatechuic acid. Two other phenols (R_F 0·41 and R_F 0·75) were present in the fluid in small quantity; these have not yet been identified. When chromatograms of oothecal fluid were sprayed with aniline hydrogen phthalate[16] a further constituent (R_F 0·18) was revealed. This substance had the same R_F value as glucose in other chromatographic solvents, namely n-butanol, (50%), ethanol (10%), water (40%) (v/v), pyridine (17%), ethyl acetate (33%), water (50%) (v/v). and phenol saturated with water in an atmosphere of NH_3. The sugar was absent in oothecal fluid that had been incubated with washed brewer's yeast at 37° for 5 min. In addition to D-glucose, three minor substances were revealed by aniline hydrogen phthalate having R_F 0·07, 0·10, 0·15. None of these substances has been investigated further.

Examination of extracts of colleterial glands

(i) *Left gland.* The isolated left glands from six animals (each animal provided about 30 mg tissue, wet wt) were homogenized with 0·2 ml ethanol in an all-glass micro-apparatus of the Potter type. Chromatography of the centrifuged extract showed one principal component (R_F 0·57) which gave positive tests for monohydroxyphenols with Pauly's diazo reagent and with ferric chloride/potassium ferricyanide. Tests for dihydroxyphenols were negative (silver nitrate, and chromaffine reagents), as were tests for reducing sugars. In addition, a minor component (R_F 0·41) of unknown composition was present. The major constituent (R_F 0·57) was isolated by elution of several chromatograms with 50% aqueous ethanol, or with water.

(ii) *Right gland.* The glands of six animals (each providing 3 mg tissue, wet wt) were homogenized in 0·1 ml water in the same apparatus as above, and the centrifuged extract was examined chromatographically. Neither in *Blatta* nor in *Periplaneta* could more than trace amounts of phenolic substances be detected. There was no substance (R_F 0·57) comparable to that in the left gland.

Interaction of extracts of right and left glands

A right and a left gland were separately homogenized as above but with water in place of ethanol, centrifuged at 2000 r.p.m. for 5 min and then the supernatant fluids were mixed and incubated at 37° for 1 hr. Chromatograms of the incubated mixture showed the presence of protocatechuic acid (R_F 0·87) (identified by any of the above reagents), and glucose (R_F 0·18). The substance (R_F 0·57) initially present in the left gland disappeared during incubation. No changes were observed when the supernatant fluid of a left gland was incubated alone.

Hydrolysis of left gland extract. The component (R_F 0·57), isolated chromatographically, was non-reducing, laevorotatory and when heated in 0·2 M H_2SO_4 at 100° for 3 hr, it released protocatechuic acid and glucose, identified chromatographically. It appeared therefore to be a β-glucoside.

Action of β-glucosidase on left gland glucoside. 1 mg chromatographically pure glucoside was mixed with 1 mg yeast β-glucosidase and incubated at 37° for 3 hr. In this time, chromatography showed that the glucoside was rapidly hydrolysed to glucose and protocatechuic acid.

[16] S. M. Partridge, *Nature, Lond.* **164**, 443, (1949).

Examination of right gland extracts. 10 mg right gland tissue was homogenized in chilled apparatus with 0·1 ml of water and the extract was centrifuged at 2000 r.p.m. for 5 min. The supernatant solution was incubated at 37° with 0·01 M-salicin (0·1 ml) and the disappearance of the substrate was followed by withdrawal of specimens of the mixture at intervals. Complete hydrolysis of the substrate into glucose and saligenin was observed within 2 hr.

Synthesis of 4-O-β-D-*glucopyranoside of protocatechuic acid*

(i) 3-*Acetylprotocatechuic acid* (I) was synthesized by the method of Lesser and Gad.[7] The ester had m.p. 198°. (Found: C, 55·0; H, 4·0. Calc. for $C_9H_8O_5$: C, 55·16; H, 4·1%).

(ii) 3-O-*Acetyl*-4-O-β-D-(*tetra*-O-*acetylglucopyranosyl*)-*protocatechuic acid* (II). The above 3-O-acetyl ester (I, 2 g) dissolved in dry quinoline (5 g) was treated with tetra-O-acetylglycosyl-α-bromide (5 g) and dry silver oxide (2 g). The mixture was shaken vigorously for 20 min, during which time the reactants became warm. The mixture was allowed to stand for 1 hr in a vacuum desiccator. It was then extracted with acetic acid (10 ml), and the filtered extract poured into iced water (100 ml). The resulting insoluble crude product was purified either by repeated recrystallization from methanol, or by absorption on charcoal. In the latter procedure, the crude product, dissolved in warm methanol, was stirred with Norite (1 g) and filtered. The charcoal was extracted repeatedly with hot methanol giving (0·2 g) of the *product* (II) as fine white needles, m.p.20 5–206°, $[\alpha]_D^{19}$ −40° ± 2 (*c*, 0·1, $CHCl_3$); light absorption maximum, 252 mμ (methanol). (Found: C, 52·1; H, 5·1. $C_{28}H_{36}O_{14}$ requires: C, 52·4; H, 5·0%).

(iii) 3-O-*Benzoyl*-4-O-β-D-(*tetra*-O-*glucopyranosyl*)-*protocatechuic acid* (III). 3-O-benzoylprotocatechuic acid (1 g, m.p. 110–112°) dissolved in dry quinoline (5 ml) was treated with silver oxide (1·5 g) and tetra-O-acetylglucopyranosyl α-bromide as above. The crude product was readily recrystallized from aqueous ethanol. Yield 0·6 g; m.p. 141–142°, $[\alpha]_D^{20}$ −35° ± 1. (*c*, 0·98, MeOH). (Found: C, 53,8; H, 5·1. $C_{28}H_{28}O_{14}2H_2O$ requires: C, 53·8; H, 5·2%).

(iv) 4-O-β-D-*glucopyranosylprotocatechuic acid* (IV). (a) The above 3-O-acetylglucoside (II, 0·1 g) was de-esterified with a catalytic amount of sodium methoxide in dry methanol as described by Zemplen and Pacsu.[17] Evaporation of the solution yielded a crystalline *product* (IV) which was recrystallized from dry ethanol. Yield 30 mg; m.p. 180–181°, $[\alpha]_D^{20}$ −59° (*c*, 0·1, MeOH), R_F 0·57 in butanol, acetic acid, water; light absorption minima at 228 mμ, 268 mμ, and maxima at 243 and 287 mμ in H_2O. (Found: C, 49·2; H, 5·0. $C_{13}H_{16}O_9$ requires: C, 49·4; H, 5·1%). Incubation of this product (2 mg) with yeast β-glucosidase at 37° results in complete hydrolysis in 45 min.

(b) The same glucoside (IV) was obtained by de-esterification of the benzoyl derivative (III) using the method of Helferich and Reischel.[10]

Acknowledgements—The authors are grateful to Professors Sir Hans Krebs and Sir Alister Hardy for their continued interest, the Agricultural Research Council for support to one of us (P. C. J. B.) and to Dr. T. Swain for helpful suggestions.

[17] G. Zemplen and E. Pacsu, *Ber. Dtsch. Chem. Ges.* **62**, 1613 (1929).