Protocatechuic Acid, the Substance Responsible for the Hardening of the Cockroach Ootheca

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The similarity between the ootheca of the cockroach (*Blatta orientalis*) and the hard brown exocuticle of insects has been already pointed out, and its investigation by one of us (Pryor, 1940*a, b*) has thrown some light on the mechanism by which it and, by inference, the insect cuticle, hardens. The ootheca, a brown bag-like structure some 8 mm. long and containing about a dozen eggs arranged in two rows, is secreted by the two colleterial glands of the female insect. The left gland secretes a water soluble protein, while the right gland produces a clear watery fluid containing a phenolic substance; it is the interaction of these two in presence of an oxidase system which causes the gradual hardening and darkening of the ootheca. The phenolic substance in the secretion of the right gland was evidently a catechol derivative from its green ferric reaction, and its solubility in aqueous sodium carbonate made it probable that it contained a carboxyl group. On this evidence it was suggested (Pryor, 1940*a*) that it might be homoprotocatechuic acid (3:4-dihydroxyphenylacetic acid), a substance whose isolation from the elytra of a beetle (*Tenebrio molitor*) has been claimed by Schmalfuss, Heider & Winkelmann (1933). However plausible this view might be it was clearly necessary to isolate and identify the phenolic substance secreted by the gland if further work on the hardening process itself was to be pursued.

Collection of material and isolation of the acid

The problem of collecting adequate material for isolation experiments was simplified by the fact that appreciable quantities of the phenolic substance are present in the fluid surrounding the eggs in the freshly laid ootheca, and that the progressive blackening of the ootheca, and consequent loss of material, is largely arrested if it is kept in absence of oxygen. For the purpose of the experiments here reported, a large colony of cockroaches was maintained and the oothecae were collected daily and stored under water, containing a small amount of cyanide and some sulphurous acid, until a sufficient

quantity had accumulated. The oothecae were crushed with aqueous potassium cyanide to inactivate the polyphenol oxidase present and extracted with acidulated water. The phenolic substance was extracted from the aqueous layer with ether and the extract treated with excess diazomethane. Hydrolysis of the product and recrystallization yielded veratric acid (3:4-dimethoxybenzoic acid), identified by m.p., mixed m.p. and preparation of its *anilide*. The obvious deduction that the substance present in the oothecae is protocatechuic acid (3:4-dihydroxybenzoic acid) was confirmed by direct isolation of this compound by a procedure similar but for the omission of the diazomethane treatment.

Hardening of the insect cuticle

The characteristic effect produced by the secretion of the right colleterial gland of the female cockroach on that of the left gland in presence of oxygen is duplicated by an aqueous solution of synthetic protocatechuic acid. As far as we are aware protocatechuic acid has not hitherto been isolated from an insect source, although it is well known as a constituent of the higher plants. Its origin remains uncertain; it might be derived from 3:4-dihydroxyphenylalanine, which has been isolated from the elytra of two species of *Melolontha* (Schmalfuss & Müller, 1927) and which is believed to be present in cockroach blood (Pryor, 1940*b*), but it should be borne in mind that there is no direct evidence for such a view, and that protocatechuic acid and 3:4-dihydroxyphenylalanine might, with at least equal probability, represent products derived by different mechanisms from some common precursor. The hardening of oothecal protein brought about by protocatechuic acid involves an oxidation of the latter and seems related to the tanning of collagen by *p*-benzoquinone (Pryor, 1940*a*), but its exact mechanism remains to be determined. The examination of other insects is planned to find whether protocatechuic acid is the normal hardening agent for the insect cuticle.

EXPERIMENTAL

Isolation of veratric acid. Some 800 oothecae of *Blatta orientalis* were crushed in dilute aqueous potassium cyanide (c. 250 ml.) containing some sulphur dioxide. After 3 hr. the mixture was filtered with the aid of 'Hyflo supercel'. The filtrate was weakly acid to litmus. The residue was extracted further by allowing to stand for 2 hr. with water (300 ml.) which had been made just acid to Congo red with sulphuric acid, and the procedure repeated until the extracts no longer gave a green colour with ferric chloride. The combined filtrate and extracts were concentrated *in vacuo* under nitrogen to small bulk (c. 300 ml.), the concentrate filtered from a trace of solid material and extracted continuously with peroxide-free ether (700 ml.). The ethereal extract which contained all the material giving a coloration with ferric chloride was concentrated to one-third bulk and treated with ethereal diazomethane (from 10 g. nitroso-*N*-methylurea). After 2 days the ether was evaporated and the residue refluxed for 2 hr. with methanolic potassium hydroxide (2%). Removal of methanol, dissolution in water and acidification of the aqueous solution gave brownish needles. Recrystallized from water this material formed colourless needles, m.p. 179–180.5°. (Found: C, 59.2; H, 5.6. Calc. for $C_9H_{10}O_4$: C, 59.3; H,

5.5%.) A mixed m.p. with authentic veratric acid (m.p. 180–181°) showed no depression. For further identification the isolated acid was converted via its acid chloride to the anilide; flat needles, m.p. 168.5°, undepressed in admixture with a specimen, m.p. 169°, prepared in similar fashion from authentic veratric acid. (Found: C, 70.2; H, 5.9; N, 5.5. Calc. for $C_{15}H_{15}O_3N$: C, 70.0; H, 5.7; N, 5.5%.) Veratric anilide is described by Bruggemann (1896) as having m.p. 154°, but this appears to be an error.

Further small amounts of product were obtained from the aqueous mother liquors after removal of the precipitated acid from the hydrolysis solution. The total yield of isolated veratric acid was 426 mg.

Isolation of protocatechuic acid. Approximately 1100 oothecae were extracted as above described, but instead of treating the ethereal solution of the phenolic material with diazomethane it was evaporated to dryness. The brownish residue recrystallized from water (charcoal) gave needles of protocatechuic acid (309 mg.), m.p. 198°, undepressed in admixture with an authentic specimen (m.p. 197.5°).

SUMMARY

The phenolic substance present in cockroach oothecae has been isolated and identified as protocatechuic acid (3:4-dihydroxybenzoic acid).

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The Spectrophotometric Determination of Tyrosine and Tryptophan in Proteins

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Solutions of most proteins show selective absorption in the ultra-violet. In each case the exact position of the maximum (near 280 $m\mu$) varies with pH. The majority of the constituent amino-acids are practically transparent in the region 250–320 $m\mu$ and it is known that phenylalanine, tyrosine and tryptophan are together responsible for the observed absorption of protein solutions. Phenylalanine, as would be expected from its constitution, shows weak absorption, which under favourable conditions

can be resolved into several narrow bands, but in 0.1N-NaOH the maximum occurs near 258.5 $m\mu$ with a low molecular extinction coefficient (c. 200). The absorption exerted by tyrosine and by tryptophan is much stronger, and as a first approximation the phenylalanine contribution can be neglected. Inasmuch as wave-lengths can be selected at which phenylalanine makes no contribution, it should be possible to treat protein solutions as two-component systems for spectrophotometric analysis.