3. α -Phenylmethylaninoctoic nitrile, and $\alpha\beta$ -bis(α' -cyano- α -heptylamino)ethane,

have aphicidal activity only slightly less than that of dodecyl thiocyanate or nicotine.

4. The mode of action of these insecticides is discussed in relation to their chemical reactivity and their physicochemical properties.

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The Occurrence of Phenolic Substances in Arthropods

BY R. H. HACKMAN, M. G. M. PRYOR AND A. R. TODD

Chemical and Zoological Laboratories, University of Cambridge

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Pryor (1940) showed that the hardening of the protein component of insect cuticle is due to the tanning action of an agent produced by oxidation of a phenolic substance. In the analogous hardening of the cockroach ootheca, Pryor, Russell & Todd (1946) showed that the phenolic substance concerned is 3:4-dihydroxybenzoic acid (I) and suggested a possible mechanism for the hardening process. It was clearly desirable to examine a number of insect species in order to establish the range of phenolic substances which might be concerned.

Polyhydric phenols appear to be widely distributed in arthropods, although very few have actually been isolated, most investigators having simply demonstrated their presence in ethanolic extracts by means of colour reactions. In this way, Pryor (1940) has shown that *o*-dihydric phenols occur in representatives of six orders of insects (Orthoptera, Odonata, Coleoptera, Hemiptera, Lepidoptera, Hymenoptera), and Lafon (1943) has found them in insects and in Arachnida (Scorpionidea, Araneida). In insects, phenols are associated with some of the layers of the epicuticle (Wigglesworth, 1947; Webb, 1947) and with the brown proteins (sclerotins) which are responsible for the hardness of the exocuticle. They also occur in the epicuticle of

arthropods, whose exocuticle is hardened with calcium carbonate; by a less specific test (the reduction of ammoniacal silver oxide, performed on paraffin sections) we have shown that they are probably present in the epicuticle of a typical diplopod (*Tachypodoiulus niger* Leach), and Dennell (1947*b*) has found them in the epicuticle of several species of Crustacea Malacostraca. Using the same test, Beament (1947) has found that some of the layers of the chorion of the eggs of *Rhodnius prolixus* Stål (Hemiptera; Reduviidae) are rich in dihydric phenols.

The earliest recorded isolation of a phenolic substance from insects is that of Villon, who is reported by Slater (1887) as having obtained 15 g. of an 'alcohol soluble tannin' from 500 g. of *Sitophilus granarius* L. Schmalfuss & Müller (1927) isolated 3:4-dihydroxyphenylalanine from the elytra of two species of *Melolontha*. Schmalfuss, Heider & Winkelmann (1933) isolated 3:4-dihydroxyphenylacetic acid (II) from the elytra of *Tenebrio molitor* L., and the same acid was obtained by Schmalfuss (1937) from the cuticles of *Cetonia aurata* L., *Potosia cuprea* F. and *Melolontha hippocastani* F. (Coleoptera; Scarabaeidae). More recently, Pryor *et al.* (1946) isolated 3:4-dihydroxybenzoic acid (protocatechuic

acid) (I) from oothecae of *Blatta orientalis*, and the same authors (Pryor *et al.* 1947) obtained 3:4-dihydroxybenzoic acid from puparia of *Calliphora erythrocephala* L. and 3:4-dihydroxyphenylacetic acid and α -hydroxy- β -(3:4-dihydroxyphenyl)-propionic acid (3:4-dihydroxyphenyllactic acid) from imagines of *Tenebrio molitor* L.

We have now examined the following species: *Locusta migratoria* L., *Rhizopertha dominica* F. (Coleoptera; Bostrychidae), *Sitophilus* (= *Calandra* auct. non Clairv.) *granarius* L., *S. oryzae* L. (Coleoptera; Curculionidae), *Tribolium confusum* J. du Val. and *T. ferrugineum* F. (= *castaneum* Herbst.) (Coleoptera; Tenebrionidae).

The experimental procedure was a modified version of that used in our previous studies and gave much higher yields of phenolic acids. The modifications included freeze drying of the insect material, followed by mechanical grinding, as well as minor changes in the methods of purification. During the isolation procedure, the phenolic acids present were methylated to facilitate working up, so that the acids were actually isolated as their methyl ethers. Previous work (Pryor *et al.* 1946, 1947) leaves no reasonable doubt that the acids present in the insects are in the unmethylated form. The results, which are recorded in the experimental portion, show that all the species examined contain 3:4-dihydroxyphenylacetic acid (II) and the *Tribolium* species contain, in addition, 3:4-dihydroxybenzoic acid (I). The low yield of 3:4-dimethoxyphenylacetic acid obtained from *Locusta* is perhaps to be ascribed to the low ratio of cuticle weight to total weight, as compared with the ratio in the other insects investigated. In addition to the insects mentioned in the experimental section we also re-examined imagines of *Tenebrio molitor*, but, in confirmation of our earlier results (Pryor *et al.* 1947), we were unable to detect in them any 3:4-dihydroxybenzoic acid.

Summarizing the information available from the present and earlier work, it appears that phenolic acids have been identified by isolation (as such or as their methyl ethers) from thirteen representative

species belonging to three orders of Insecta, whilst colour reactions indicate the presence of similar *o*-dihydroxyphenolic substances in four additional orders. We feel justified, therefore, in assuming that these phenolic acids are of general occurrence and may all be concerned in cuticle hardening. Although the presence of traces of acids other than those identified cannot be wholly excluded, the pure methylated substances isolated by us accounted for more than 75% of the crude methylated phenolic extracts, so that the distribution of the identified acids is of some interest. Of the thirteen species so far examined, ten contained 3:4-dihydroxyphenylacetic acid, which in seven cases was the only acid present; in two cases it was accompanied by 3:4-dihydroxybenzoic acid, and in one by α -hydroxy- β -(3:4-dihydroxyphenyl)-propionic acid. 3:4-Dihydroxybenzoic acid occurred alone in two species. In addition, 3:4-dihydroxyphenylalanine has been isolated from two species. The most likely mode of participation of such *o*-dihydroxy aromatic acids in cuticle hardening is enzymic oxidation followed by condensation of the oxidized material with free amino groups in the cuticular protein, so that stable cross-linked structures are formed in which the nitrogen of the amino groups becomes directly attached to the aromatic nuclei. Thiol groups in the protein may, of course, also take part in reaction with the oxidized dihydroxy acid. The process, indeed, bears a close resemblance to the tanning of leather with quinones. Catechol and 3:4-dihydroxybenzoic acid and their homologues when oxidized condense *in vitro* with compounds containing free amino groups to give compounds in which nitrogen is directly attached to the aromatic nucleus. Reactions of this type are being studied at present as an aspect of the work here described and will be reported upon in due course. As to the origin of the phenolic acids in insects, the most probable explanation is that they are degradation products of 3:4-dihydroxyphenylalanine, itself produced from tyrosine under the influence of a polyphenol oxidase. It is of interest to note that both Fraenkel & Rudall (1947) and Dennell (1947*a*) have also expressed the view that the phenol hardening in blowfly puparia is brought about by a phenolic material produced from blood tyrosine by enzymic oxidation.

Since it was desired to make our investigations as comprehensive as possible, the solutions of sulphurous acid containing cyanide in which the insects were killed and stored were always examined for the presence of phenolic substances. In the case of the two *Tribolium* species, but in no others, ether extracts of the acidic liquor, after removal of the dead insects, yielded large quantities of ethylhydroquinone (1.4% on freeze-dried insect material) identified by analysis, mixed melting point and preparation of the *dibenzoate*. This substantially con-

firms the findings of Alexander & Barton (1943), who examined the volatile secretion of the flour beetles, *Tribolium confusum* J. du Val. and *T. ferrugineum* F. (= *castaneum* Herbst.), which causes flour infested by these beetles to become pink in colour and unpalatable; they concluded on the evidence available to them that the active principle was ethyl *p*-benzoquinone. Presumably in our experiments the insects, on being dropped into the sulphurous acid solution, eject ethyl *p*-benzoquinone which is then reduced to the hydroquinone. Quinones have previously been recorded in secretions of the stink glands of other arthropods. Béhal & Physalix (1900) described a compound with the properties of a *p*-quinone in the lateral stink glands of the millipede, *Schizophyllum mediterraneum* Latzel, but did not isolate it; similar observations on other diplopods are described by Burt (1947). Ethyl *p*-benzoquinone is probably also the chief constituent of the yellow oils obtained by Palm (1946) from stink glands on the prothorax and on the ventral side of the tip of the abdomen of *Aphanotus destructor* Uytt. and *Tribolium confusum* J. du Val.

The crude crystalline material first obtained by us during the isolation of ethylhydroquinone had a low melting point and was difficult to purify. Careful fractionation of crystallization mother liquors obtained in purifying the ethylhydroquinone and its dibenzoate yielded products which, although melting lower than methylhydroquinone (toluhydroquinone) and its dibenzoate, showed no depression in melting point when mixed with authentic specimens of these materials, but were depressed in melting point by pure ethylhydroquinone and its dibenzoate. From these facts and the elementary analysis, we conclude that the extract contained small amounts of toluhydroquinone. It is probable that the similar difficulty experienced by Alexander & Barton (1943) in purifying their material was due to the presence in the volatile secretion of small amounts of methyl *p*-benzoquinone (toluquinone) as well as ethyl *p*-benzoquinone.

The most probable precursor of these *p*-quinones would seem to be 2:5-dihydroxyphenylalanine, an acid already suggested as an intermediate in the formation of homogentisic acid (2:5-dihydroxyphenylacetic acid) in human alcaptonuria (Neuberger, Rimington & Wilson, 1947). 2:5-Dihydroxyphenylalanine itself has not been found in nature, but its production from tyrosine (cf. Blaschko & Sloane Stanley, 1948) by oxidation as an alternative to 3:4-dihydroxyphenylalanine would seem reasonable enough. Such an oxidation would be analogous to the well-known formation of toluhydroquinone on oxidizing *p*-cresol with potassium persulphate in acid solution, rearrangement with migration of the methyl group occurring in the process (Kumogi & Wolfenstein, 1908); a similar

migration of the aminopropionic acid side chain during the oxidation of tyrosine would yield 2:5-dihydroxyphenylalanine, which might then be degraded to ethylhydroquinone and toluhydroquinone. Wolkow & Baumann (1891) have already shown that the intestinal bacteria in dogs can convert homogentisic acid to toluhydroquinone.

EXPERIMENTAL

Methylated phenolic acids from insects

The insects were collected and stored in water containing KCN to inactivate polyphenol oxidases, and SO₂ to prevent aerial oxidation of hydroquinone or catechol derivatives. Before working up, the material (whole insects, except in the case of *Locusta* where only the heads were used) was separated from the liquid, freeze dried and ground in a mechanical mortar. It was then extracted with methanol in a vapour-jacketed Soxhlet for 24 hr., filtered, the methanolic extracts (which had a blue fluorescence) evaporated to dryness under reduced pressure in an atmosphere of N₂ and the residue extracted with water until the extracts gave a negative reaction with FeCl₃. The combined aqueous extracts were acidified to congo red with H₂SO₄, extracted continuously with peroxide-free ether, the ethereal extract concentrated to c. 100 ml. and the phenols present methylated by addition of ethereal diazomethane (from 20 g. nitrosomethylurea). The solution so obtained was left overnight, solvents removed by evaporation and the residual oil refluxed for 2 hr. with methanolic KOH (50 ml. of 2%) to hydrolyze ester groups. After removal of methanol, water was added, the solution acidified with H₂SO₄ and extracted with ether. The ethereal extract was shaken with aqueous Na₂CO₃ to remove acids, and the aqueous layer was again acidified and extracted with ether. The ethereal extract dried over Na₂SO₄ and evaporated yielded usually a semi-solid mass. Fractional crystallization, or, where only one acid was present, simple recrystallization, from a mixture of benzene and light petroleum yielded the pure methylated acids. The products obtained in each case are given below:

Locusta migratoria. 3:4-Dimethoxyphenylacetic acid, m.p. 97°, undepressed by an authentic specimen, m.p. 98° (Found: C, 61.3; H, 6.4. Calc. for C₁₀H₁₂O₄: C, 61.2; H, 6.2%). Yield, 0.2% on dried material (415 g.).

Rhizopertha dominica. 3:4-Dimethoxyphenylacetic acid, m.p. and mixed m.p. 98° (Found: C, 61.9; H, 6.0%). Yield, 1.5% on dried material (30 g.).

Sitophilus granarius. 3:4-Dimethoxyphenylacetic acid, m.p. and mixed m.p. 96° (Found: C, 61.3; H, 6.0%). Yield, 1.8% on dried material (101 g.).

S. oryzae. 3:4-Dimethoxyphenylacetic acid, m.p. and mixed m.p. 97° (Found: C, 60.9; H, 5.9%). Yield, 1.4% on dried material (128 g.).

Tribolium confusum. 3:4-Dimethoxyphenylacetic acid (yield, 1.4%), m.p. and mixed m.p. 96° (Found: C, 61.1; H, 6.4%), together with 3:4-dimethoxybenzoic acid (yield, 0.4%), m.p. 179°, undepressed by an authentic specimen, m.p. 180° (Found: C, 59.3; H, 5.7. Calc. for C₉H₁₀O₄: C, 59.3; H, 5.5%). The amount of dried insects used was 45 g.

T. ferrugineum. 3:4-Dimethoxyphenylacetic acid (yield, 1.9%), m.p. and mixed m.p. 98° (Found: C, 61.0; H, 5.9%),

together with 3:4-dimethoxybenzoic acid (yield, 0.5%), m.p. and mixed m.p. 180° (Found: C, 59.1; H, 5.6%). The amount of dried insects used was 57 g.

Isolation of ethylhydroquinone (1:4-dihydroxy-2-ethylbenzene)

The acidic liquid from the *Tribolium* species, after separation of the insect bodies (dry wt. 100 g.), was continuously extracted with ether for 24 hr., and the ethereal extract dried over Na_2SO_4 and evaporated. The residual oil was now treated with an excess of concentrated aqueous FeCl_3 solution and quinones formed were collected by steam distillation. The steam distillate was saturated with SO_2 and extracted with ether. Evaporation of the ethereal extract gave the crude ethylhydroquinone as a colourless crystalline solid, m.p. 106°, unaltered by repeated recrystallization from benzene or chloroform. After several recrystallizations from water (charcoal) the product formed colourless plates, m.p. 114°, alone or in admixture with authentic ethylhydroquinone (m.p. 114°) prepared by Clemmensen reduction of 2:5-dihydroxyacetophenone (Found: C, 69.9; H, 7.5. Calc. for $\text{C}_{11}\text{H}_{10}\text{O}_2$: C, 69.6; H, 7.3%). For further confirmation of its identity, the product was treated with excess of benzoyl chloride in pyridine. The dibenzoate so formed separated from methanol in colourless needles, m.p. 89°, undepressed in admixture with authentic dibenzoate of ethylhydroquinone (m.p. 89°) prepared as described below (Found: C, 76.1; H, 5.0. Calc. for $\text{C}_{22}\text{H}_{18}\text{O}_4$: C, 76.2; H, 5.2%).

Concentration of the aqueous crystallization mother liquors of the ethylhydroquinone yielded a crop of material which, after further fractionation from water and recrystallization from benzene, had m.p. 114°, undepressed in admixture with toluhydroquinone (m.p. 124°), but depressed in admixture with ethylhydroquinone (m.p. 114°). Similarly, fractional crystallization from methanol of the dibenzoate prepared from the crude ethylhydroquinone, yielded a small amount of material, m.p. 93°, undepressed in admixture with the dibenzoate of toluhydroquinone

(m.p. 122°), but depressed in admixture with the dibenzoate of ethylhydroquinone (m.p. 89°) (Found: C, 76.1; H, 5.1. Calc. for $\text{C}_{21}\text{H}_{16}\text{O}_4$: C, 75.9; H, 4.85. Calc. for $\text{C}_{22}\text{H}_{18}\text{O}_4$: C, 76.2; H, 5.2%).

Ethylhydroquinone dibenzoate. Benzoyl chloride (1 ml.) was added to a solution of ethylhydroquinone (0.5 g.) in dry pyridine (10 ml.). Reaction set in at once and the mixture was allowed to stand overnight and then poured on a mixture of ice and dilute H_2SO_4 and extracted with ether. The ethereal extract was thoroughly washed, dried over Na_2SO_4 and evaporated. Recrystallization of the solid residue from methanol (charcoal) gave *ethylhydroquinone dibenzoate* as colourless needles, m.p. 89° (Found: C, 76.2; H, 5.3. $\text{C}_{22}\text{H}_{18}\text{O}_4$ requires C, 76.2; H, 5.2%).

SUMMARY

1. The occurrence of 3:4-dihydroxyphenylacetic acid in six insect species is reported. Two of these species contain, in addition, 3:4-dihydroxybenzoic acid. These phenolic acids are believed to play a part in the hardening of insect cuticle.

2. From aqueous sulphurous acid used to kill and store two species of *Tribolium*, ethylhydroquinone has been isolated in substantial yield. Its isolation confirms the results of earlier workers who showed that ethyl *p*-benzoquinone is secreted by these insects. Evidence is presented that toluhydroquinone is also present in the extracts.

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